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A class of new synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) effectively induce apoptosis of prostate cancer cells. Our previous study demonstrated that AHPN/CD437-induced apoptosis of LNCaP prostate cancer cells requires the expression of TR3 (also called nur77 or NGFI-B) that is an orphan member of the steroid/thyroid/retinoid receptor superfamily and its nuclear export and mitochondrial localization. In studying how TR3 nuclear export, mitochondrial targeting and apoptosis induction in prostate cancer cells are regulated, we have demonstrated that the migration of TR3 from the nucleus to the cytoplasm requires retinoid X receptor (RXR) through their heterodimerization. In addition, we show that the TR3 cytoplasmic localization and apoptotic effect are inhibited by RXR ligand 9-cis retinoic acid. Moreover, we demonstrate that TR3 interacts with Bcl-2 and that the interaction is essential for TR3 to target mitochondria and to induce apoptosis. These data not only enhance our understanding the molecular mechanism by which TR3 nuclear export, mitochondrial targeting and apoptosis induction are regulated but also provide important information for developing novel strategies for inducing apoptosis of prostate cancer cells.

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INTRODUCTION

Prostate cancer is the second most common cause of male cancer death in the West. Conventional chemotherapy and radiotherapy are still of limited effectiveness. Recent progress has suggested that induction of cancer cell death is a plausible way to restrict tumor growth, and many chemotherapeutic drugs induce death of cancer cell. Retinoids, vitamin A and its analogs, are known to induce death of prostate cancer cells. However, the effectiveness of conventional vitamin A derivatives, such as all-*trans* retinoic acid, is limited to androgen-dependent prostate cancer cells. Recently, a new class of synthetic vitamin A derivatives related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) have been found to effectively induce death of both androgen dependent and -independent prostate cancer cells (1-3), suggesting that they represent a new class of chemotherapeutic agents for treating advanced hormone refractory prostate cancer. We subsequently showed that TR3/nur77, an orphan member of the steroid/thyroid/retinoid receptor superfamily, is required for induction of death of both androgen-dependent and -independent prostate cancer cells by AHPN/CD437 and other death-inducing agents (3). Moreover, we discovered that TR3/nur77, in response to AHPN/CD437 and other apoptotic stimuli, migrated from the nucleus to the cytoplasm, where it targeted mitochondria, resulting in cytochrome c release and apoptosis (3).

This application focuses on the molecular mechanism by which TR3-mediated apoptosis of prostate cancer cells is mediated and regulated. In the proposed studies, we plan to investigate whether this novel nuclear-to-mitochondrial pathway for apoptosis can be extended to other members of the TR3/nur77 family and to other prostate cancer cell lines, especially the hormone-refractory prostate cancer cells. In addition, we will study the mechanism by which translocation of TR3/nur77 from the nucleus to cytoplasm is regulated. Furthermore, we will study physical interactions between TR3/nur77 and members of the Bcl-2 family. Results from these studies will enhance our understanding of the mechanism by which TR3/nur77 induces apoptosis of androgen-dependent and -independent prostate cancer cells and of the molecular control of prostate cancer growth. They should provide a molecular basis for the identification of agents that induce association of TR3/nur77 with mitochondria, resulting in possible conversion of TR3/nur77 from a growth promoting to death inducing molecule in prostate cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

In the past funding year, we conducted various experiments to address the specific aims proposed in the grant application as described below.

1. Regulation of TR3 nuclear export, mitochondrial targeting and apoptosis by RXR and its ligands

TR3 is known to heterodimerize with RXR (4,5). Several experiments were conducted to determine the effect of RXR and its ligands on TR3 nuclear export, mitochondrial localization and its induced apoptosis. In LNCaP cells, RXR was found exclusively in the nucleus in the absence of treatment. However, when cells were treated with apoptotic stimulus TPA, RXR tranlocated to the cytoplasm, where it was colocalizaed with mitochondria (Figure 1A).

Pretreatment of cells with RXR agonists 9-*cis* RA or SR11237 prevented RXR mitochondrial localization. The localization of RXR on mitochondria was also demonstrated by immunoblotting analysis showing that RXR was accumulated in the mitochondria-enriched heavy membrane fraction when cells were treated with TPA or MM11453. Pretreatment of cells with 9-*cis* RA inhibited the accumulation of RXR (Figure 1C). The mitochondrial localization of RXR is depended on TR3 expression as RXR failed to reside on mitochondria in cells expressing TR3 antisense RNA (Figure 1C), suggesting that RXR may target mitochondria as TR3/RXR heterodimer. 9-cis RA also prevented TPA-induced TR3 cytoplasmic localization and cytochrome c release in LNCaP cells (Figure 1D).

Pretreatment of LNCaP cells with 9-*cis* RA or MM11237 also prevented release of cytochrome c from mitochondria and apoptosis induced by TPA and MM11453 in LNCaP cells (Figure 2).

Together, our studies demonstrate that TR3 translocates from the nucleus to the cytoplasm as TR3/RXR heterodimer and that a nuclear export sequence in the RXR is required for the process.

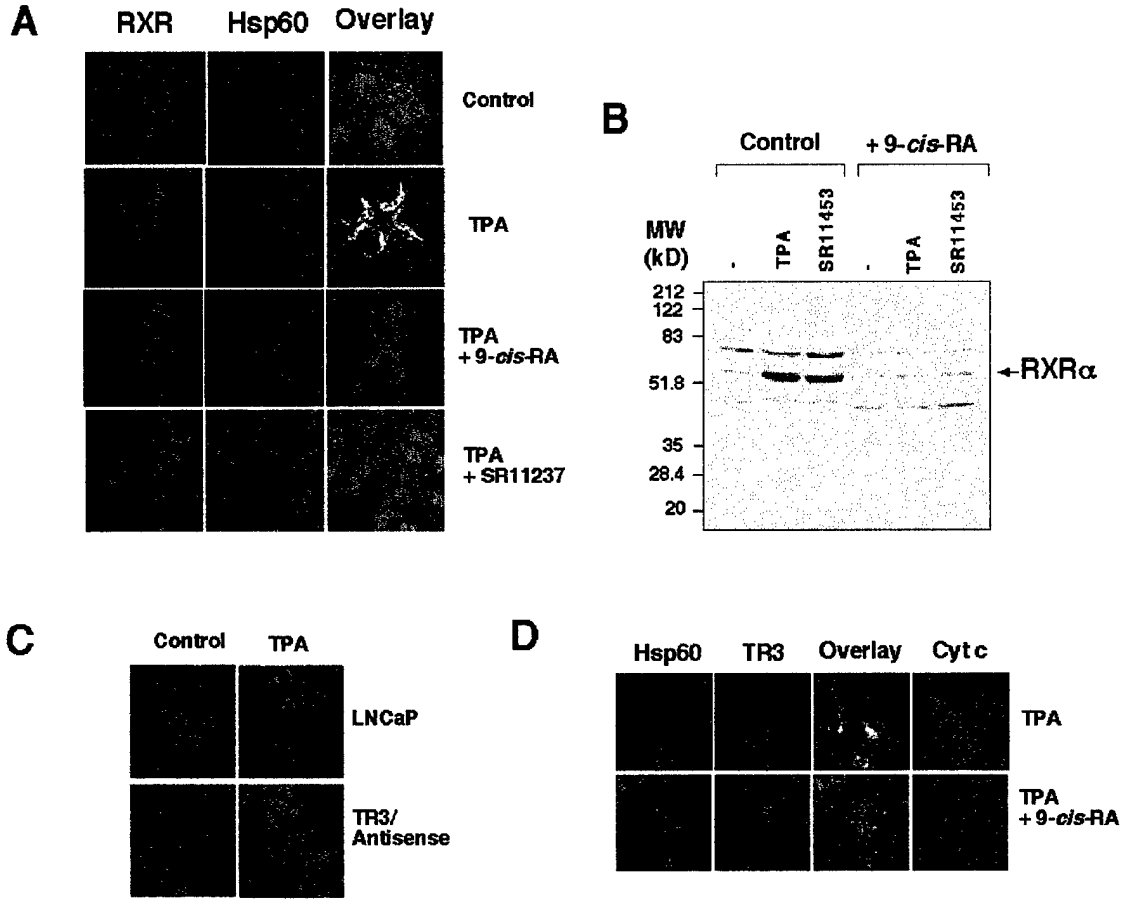

Figure 1. Localization of RXR in mitochondria in a TR3-dependent manner. A. Regulation of RXR mitochondrial localization by apoptosis inducer TPA and RXR ligands. LNCaP cells were pre-

treated with or without RXR ligands 9-*cis* RA (10^{-7} M) or SR11237 (10^{-6}M) for 12 hr before TPA treatment (1 hr), then immuno-stained with anti-RXR antibody followed by Cy3-conjugated secondary antibody (Sigma) to detect RXR, or with anti-Hsp60 followed by Cy5-conjugated secondary antibody (Sigma) to detect mitochondria. RXR and mitochondria (Hsp60) were visualized using confocal microscopy and the two images were overlaid (overlay). **B**. Apoptotic stimuli induce accumulation of RXR in mitochondria. LNCaP cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) for 3 hr in the absence or presence of 9-*cis* RA (10^{-6} M), and the HM fraction was analyzed for expression of RXR by Western blotting. **C**. Mitochondrial localization of RXR is TR3 dependent. LNCaP cells or LNCaP cells stably expressing TR3 antisense RNA (TR3/Antisense) were treated with or without TPA for 1 hr, then immuno-stained with anti-RXR antibody followed by Cy3-conjugated secondary antibody (Sigma) to detect RXR. **D**. 9-cis RA inhibits TPA-induced TR3 mitochondrial localizaion and cytochrome c release in LNCaP cells.

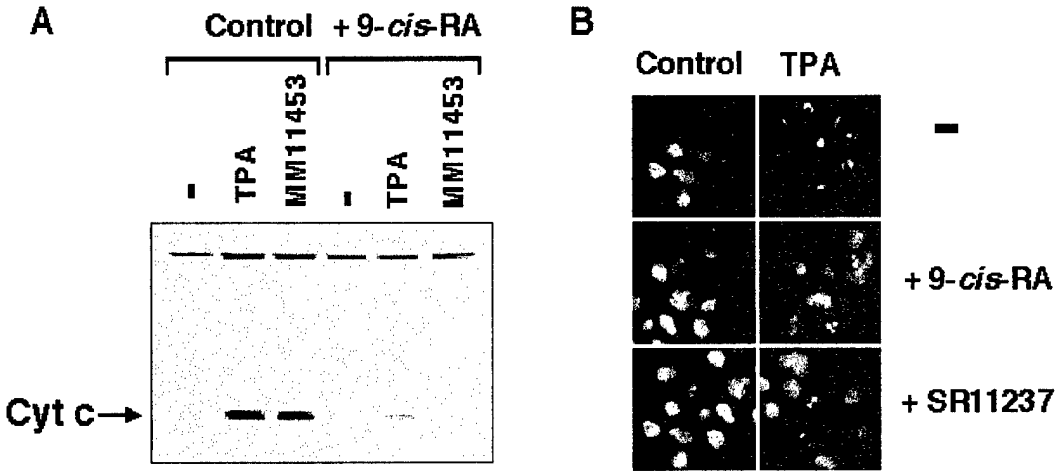

Figure 2. Inhibition of cytochrome c release and apoptosis by RXR agonists. A. Inhibtion of cytochrome c release. LNCaP cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) I the presence or absence of 9-*cis* RA (10^{-7} M) for 3 hours. Cytosolic fractions were then prepared and analyzed for expression of cytochrome c (Cyt c) by Western blotting. A nonspecific band at ~ 70 kd served as a control for equal loading of proteins. **B**. Inhibition of apoptosis. LNCaP cells were treated with MM002 (10^{-6} M) in the presence or absence of 9-*cis* RA (10^{-6} M) or SR11237 (10^{-6}M) for 36 hours and nuclei were stained by DAPI.

2. Interaction between TR3 and Bcl-2

Members of the Bcl-2 family are important regulators of cell death and survival (6,7). Many of which, such as Bcl-2, are located predominantly in the outer mitochondrial membrane (6,7). We investigated the possibility that TR3 targeted mitochondria by interacting with Bcl-2 that is known to reside on the outer membrane of mitochondria. TR3/ΔDBD, which constitutively resides on mitochondria (3), was analyzed in a co-immunoprecipitation (Co-IP) assay for its interaction with Bcl-2 by transfecting it into human embryonic kidney cell line 293T alone or with Bcl-2 expression vector. Co-IP assay showed that a significant amount of TR3/ΔDBD was co-precipitated with Bcl-2 by anti-Bcl-2 antibody (Figure 3a). In a reporter gene assay in CV-1 cells, transactivation of TR3 on its responsive element (NurRE-tk-CAT) was

potently inhibited by cotransfection of Bcl-2, but not by Bax (Figure 3b). The TR3/ΔDBD and Bcl-2 interaction was also demonstrated by the GST-pull down assay, showing that ^{35}S-labeled Bcl-2 was pulled down by GST-TR3 but not by GST (Figure 3c). Transfected Bcl-2 and TR3/ΔDBD colocalized in LNCaP cells as revealed by confocal microscopy analysis (Figure 3d). Thus, TR3 interacts specifically with Bcl-2.

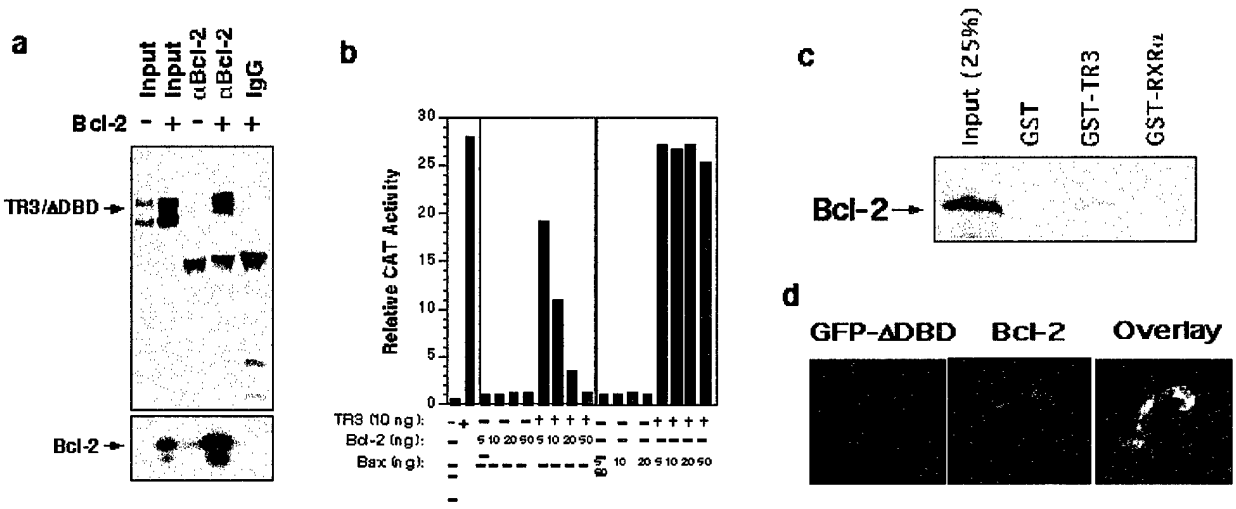

Figure 3. Interaction of TR3 with Bcl-2. a. *In vivo* Co-IP assay. GFP-TR3/ΔDBD was cotransfected alone or with Bcl-2 expression vector into 293T cells. The expressed GFP-TR3/ΔDBD mutant protein was then precipitated by using either anti-Bcl-2 antibody or control IgG and detected by western blotting using anti-GFP antibody. The same membranes were also blotted with anti-Bcl-2 antibody to determine precipitation specificity and efficiency. Input represents 10% of total cell extract used in the precipitation assays. **b.** Inhibition of TR3-dependent transactivation by Bcl-2. CV-1 cells were transfected with the NurRE-*tk*-CAT (3) with or without TR3 expression vector (25 ng) together with or without the indicated amount of Bcl-2 or Bax expression vector. CAT activity was then determined. **c.** GST-pull down of Bcl-2 by TR3. GST-TR3, GST or GST-RXR immobilized on 20 μl of glutathione-Sepharose was incubated with 10 μl of *in vitro* synthesized ^{35}S-labeled Bcl-2. Bound proteins were analyzed by SDS-PAGE autoradiography. **d.** Confocal microscopy analysis. Expression vectors for GFP–TR3/ΔDBD and Bcl-2 were cotransfected into LNCaP cells. After 20 h, cells were immunostained with anti-Bcl-2 antibody then Cy3-conjugated secondary antibodies (Sigma). GFP–fusion and Bcl-2 were visualized using confocal microscopy.

A unique interaction between TR3 and Bcl-2. On analysis of TR3 mutants (Figure 4a) by Co-IP, we found that the C-terminal domain (DC3), but not the N-terminal domain (N168), of TR3/ΔDBD, bound Bcl-2 (Figure 4b). A 69 amino acid C-terminal fragment (DC1) strongly interacted with Bcl-2, whereas deletion of DC1 from TR3/ΔDBD (TR3/ΔDBD /ΔDC1) largely abolished interaction with Bcl-2 (Figure 4b). Binding of the BH3 domain to Bcl-2 is mediated by a hydrophobic cleft in Bcl-2 formed by its BH1, BH2, and BH3. To determine whether TR3/ΔDBD bound to the Bcl-2 hydrophobic groove, several Bcl-2 mutants with mutation of amino acids, Tyr^{108}, Leu^{137}, or Arg^{146}, critical for the formation of the hydrophobic cleft were constructed and analyzed for their interaction with TR3/ΔDBD. These mutants,Y108KBcl-2, L137Abcl-2, and R146QBcl-2, failed to interact with Bax as expected (data not shown). However, they were still capable of binding to TR3/ΔDBD (Figure 4c). Moreover, a BH3-only Bcl-2 family protein Bcl-Gs (8) did not compete with DC1 for binding Bcl-2 (Figure 4d). Surprisingly, it enhanced the binding of DC1 to Bcl-2. The enhancing effect required its binding to Bcl-2 since mutant Bcl-Gs (L216EBcl-Gs) with mutation in its BH3 domain, which abolishes

its ability of binding Bcl-2,failed to enhance DC1 binding to Bcl-2. Thus, TR3 interacts with Bcl-2 in a manner that is different from other Bcl-2 family proteins.

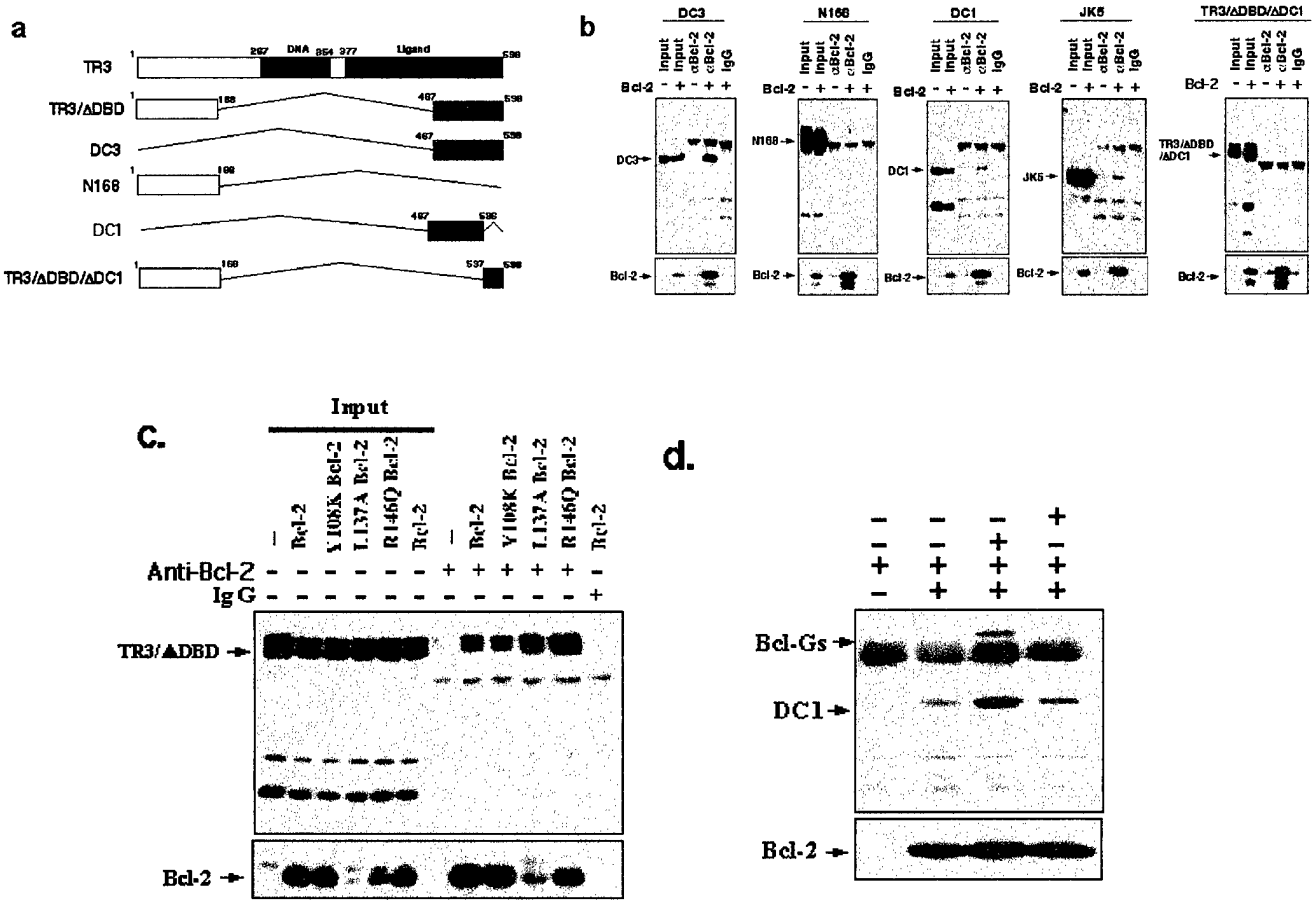

Figure 4. Mutational analysis of TR3 and Bcl-2. a. Schematic representation of TR3 mutants. DNA-binding and ligand-binding domains of TR3 are indicated. **b**. *In vivo* Co-IP. The indicated TR3 mutants were analyzed for their interaction with Bcl-2 by Co-IP assay as described in Fig. 3a. **c.** The hydrophobic groove of Bcl-2 is not required for its binding to TR3/ΔDBD. Bcl-2 mutant, Y108 KBcl-2, L137A Bcl-2 or R146QBcl-2, was analyzed for their interaction with GFP-TR3/ΔDBD in 293T cells by Co-IP as described in Fig 3a. **d.** Competition assay. Bcl-2 was co-transfected into 293T cells with either GFP-DC1 or GFP-Bcl-Gs or a Bcl-Gs mutant and the Co-IP was performed as described in Fig. 3a.

Bcl-2 mediates TR3 mitochondrial targeting and apoptosis. We studied whether TR3/ΔDBD targets mitochondria via its interaction with Bcl-2. TR3/ΔDBD expressed in 293T cells exhibited a diffused distribution pattern (Figure 5a), but colocalized with Bcl-2 and heat shock protein 60 (Hsp60), a mitochondrial specific protein when Bcl-2 and TR3/Δ DBD were coexpressed. (Figure 5a). Immunoblotting of the mitochondria-enriched heavy membrane (HM) fractions showed a significantly enhanced accumulation of TR3/ΔDBD in mitochondria when Bcl-2 was coexpressed (Figure 5b). These results demonstrate that Bcl-2 acts as a receptor for TR3 to target mitochondria. We next studied the involvement of TR3/Bcl-2 interaction in TR3-induced cyto c release. In the absence of Bcl-2 cotransfection, TR3/ΔDBD did not release cyto c from mitochondria as determined by confocal microscopy analysis (Figure 5c). However, on

Bcl-2 cotransfection TR3/ΔDBD and Bcl-2 colocalized and cyto c was released (Figure 5c). DAPI staining indicated that TR3ΔDBD only caused nuclear fragmentation or condensation when Bcl-2 was cotransfected (Figure 5d). Thus, the TR3ΔDBD-Bcl-2 interaction is required to induce cyto c release and apoptosis.

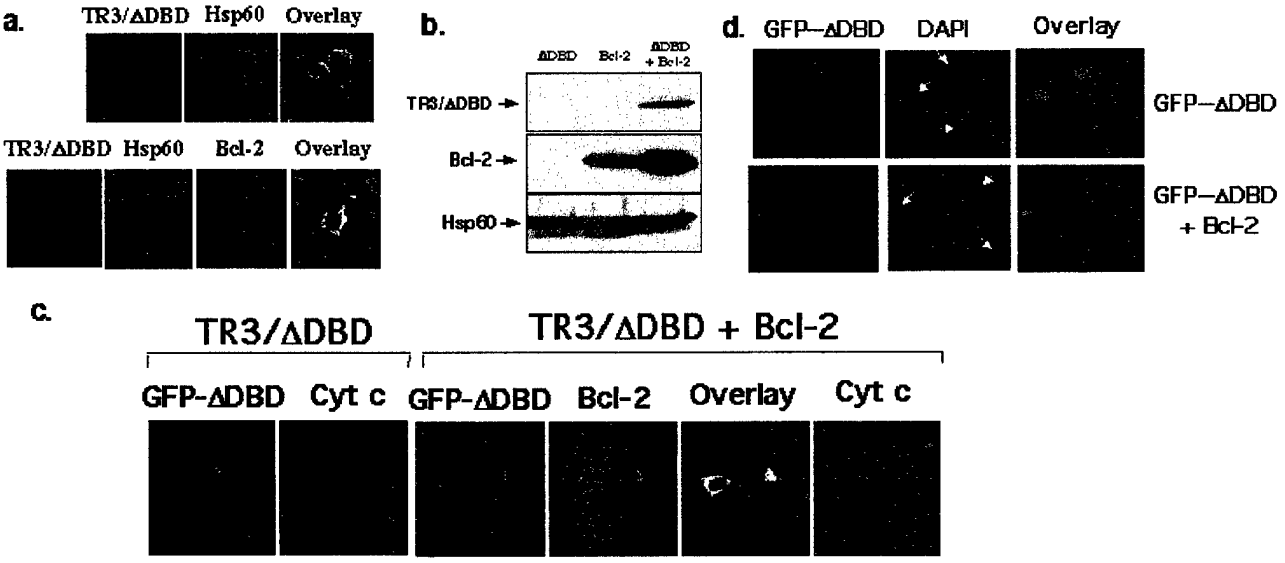

Figure 5. Bcl-2 expression promotes TR3/ΔDBD mitochondrial localizationn and its apoptotic effect. a. Confocal microscopy analysis. GFP-TR3ΔDBD and Bcl-2 were transfected into 293T cells alone or together. Cells were immunostained with anti-Bcl-2 antibody followed by Cy3-conjugated secondary antibody, or with anti-Hsp60 antibodyfollowed by Cy5-conjugated secondary antibody. Bcl-2, TR3/ΔDBD and mitochondria (Hsp60) were visualized using confocal microscopy. **b**. Western blotting. The heavy membrane fractions were prepared and analyzed for accumulation of TR3/ΔDBD by Western blotting using anti-GFP antibody. **c**. Bcl-2 is required for TR3 to induce cyto c release. GFP-TR3/ΔDBD and Bcl-2 were transfected into 293T cells alone or together. Cells were immunostained with anti-cyto c (cyt c) antibody followed by Cy5-conjugated secondary antibody, or with anti-Hsp60 followed by Cy3-conjugated secondary antibody. Cyto c, TR3/ΔDBD, and mitochondria (Hsp60) were visualized using confocal microscopy. **d**. Bcl-2 is required for TR3/ΔDBD to induce apoptosis. Nuclei of 293T cells transfected with GFP-TR3/ΔDBD alone or together with Bcl-2 were stained by DAPI. GFP-ΔDBD expression and nuclear morphology were visualized by fluorescence microscopy. Arrows indicate cells displaying nuclear condensation and fragmentation.

REPORTABLE OUTCOMES

1. Dawson, M.I., Hobbs, P., Peterson, V., Leid, M., Lange, C., Feng, K., Chen, G., Gu, J., Li, H., Kolluri, S., Zhang, X-k., Zhang, Y., and Fontana, J. Induction of apoptosis in cancer cells by a novel analog of 6-[3- (1-Adamantyl) –4- hydroxyphenyl} –2- naphthalenecarboxylic acid (AHPN) lacking retinoid receptor transcriptional activation activity. Cancer Research. 61:4723-4730, 2001.
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CONCLUSIONS

In summary, we have conducted enormous amount of experiments to study the regulation of TR3 nuclear export, mitochondrial targeting and apoptosis induction in prostate cancer cells. Our results demonstrate that the migration of TR3 from the nucleus to the cytoplasm requires RXR through their heterodimerization. In addition, we show that the TR3 cytoplasmic localization and apoptotic effect are regulated by RXR ligands. These data not only enhance our understanding the molecular mechanism of TR3 nuclear export but also provide important information for developing RXR ligands for regulating apoptosis of prostate cancer cells. In addition, we show that TR3 interacts with Bcl-2 and that the interaction between TR3 and Bcl-2 is essential for TR3 to target mitochondria and to induce apoptosis. Our observation that the TR3-Bcl-2 interaction converts Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule provides novel molecular basis for inducing apoptosis of Bcl-2-expressing cancer cells. The overall plan for the next year of research remains the same as proposed in the original grant application.

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APPENDICES

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[CANCER RESEARCH 61, 4723–4730, June 15, 2001]

Apoptosis Induction in Cancer Cells by a Novel Analogue of 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic Acid Lacking Retinoid Receptor Transcriptional Activation Activity[1]

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ABSTRACT

The retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN) is reported to have anticancer activity *in vivo*. Induction of cell cycle arrest and apoptosis in cancer cell lines refractory to standard retinoids suggests a retinoid-independent mechanism of action for AHPN. Conformational studies suggested that binding of AHPN does not induce an unusual conformation in retinoic acid receptor (RAR) γ. The 3-chloro AHPN analogue MM11453 inhibited the growth of both retinoid-resistant (HL-60R leukemia, MDA-MB-231 breast, and H292 lung) and retinoid-sensitive (MCF-7 breast, LNCaP prostate, and H460 lung) cancer cell lines by inducing apoptosis at similar concentrations. Before apoptosis, MM11453 induced transcription factor TR3 expression and loss of mitochondrial membrane potential characteristic of apoptosis. MM11453 lacked the ability to significantly activate RARs and retinoid X receptor α to initiate $(TREpal)_2$-*tk*-CAT reporter transcription. These results, differential proteolysis-sensitivity assays, and glutathione *S*-transferase-pulldown experiments demonstrate that, unlike AHPN or the natural or standard synthetic retinoids, MM11453 does not behave as a RAR or retinoid X receptor α transcriptional agonist. These studies strongly suggest that AHPN exerts its cell cycle arrest and apoptotic activity by a signaling pathway independent of retinoid receptor activation.

INTRODUCTION

The natural RAs[3] and their synthetic analogues are being investigated as chemotherapeutic agents because they inhibit proliferation, induce apoptosis in cancer cells, and retard tumor xenograft growth (1). These standard retinoids exert their antiproliferative effects by influencing the transcriptional activity of RAR and RXR subtypes α, β, and γ (reviewed in Ref. 2). Retinoids complexed to a RXR/RAR can activate or repress gene transcription from RA response elements in the promoter of retinoid-sensitive genes. A retinoid bound to an RXR can modulate activation by other transcription factors with which it dimerizes (2). Retinoid receptor-ligand complexes also compete with other transcription factors for coactivator proteins (3, 4), whereas nonliganded dimers compete for corepressors (5).

The diversity from the six subtypes and variations in their expression patterns (2, 6–9), response element sequences, intermediary proteins, and other transcription factors (2) led to the identification of receptor-selective retinoids to enhance efficacy by reducing the systemic toxicity associated with retinoids activating all receptors (10). Receptor class and subtype-selective compounds (reviewed in Refs. 1 and 11) also provide a means for studying individual receptor-signaling pathways.

On evaluating RARγ-selective retinoids, we observed that AHPN (CD437 [1] in Fig. 1; Ref. 12) rapidly caused detachment of retinoid-sensitive MCF-7 breast and NIH:OVCAR-3 ovarian cancer cells (13, 14). This atypical retinoid activity extended to retinoid-resistant lines, including MDA-MB-231 breast cancer and HL-60R leukemia (13). AHPN induced cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ expression (13), G_0-G_1 cell cycle arrest (13), and apoptotic events, such as caspase activation, *gadd45* expression (15), poly(adenosyl diphosphate-ribose) polymerase cleavage, and DNA fragmentation (13). Interestingly, apoptosis occurred in the absence of functional tumor suppressor *p53* (13), the gene for which is mutated in many cancers (16). Apoptosis by AHPN and its derivatives and analogues was subsequently observed in other lines derived from tumors and their metastases (17–25).

The efficacy against retinoid-resistant cancer cells prompted studies on how AHPN induces apoptosis. To reduce complications, we conducted mechanistic studies in cells lacking functional retinoid receptors (26) and used apoptotic AHPN analogues lacking retinoid agonist transactivation activity, such as MM11453 [2]. MM11453 induced apoptosis by a cascade that included mitochondrial translocation of transcription factor TR3/nur77/NGFIB-II (TR3), cytochrome *c* release, caspase activation, and DNA fragmentation (27). Binding of MM11453 to RARs and RXRα did not cause the conformational changes of AHPN that led to corepressor loss and coactivator recruitment. We report here the characterization and anticancer activity of MM11453, the prototype for new nonretinoidal apoptotic agents with potential for cancer treatment.

MATERIALS AND METHODS

Retinoids. AHPN [1] was prepared by modifying a reported procedure (28). AHPN (MM11453) [2] was synthesized as follows. The biaryl bond was introduced by palladium(0)-catalyzed coupling between 3-(1-adamantyl)-4-benzyloxybenzeneboronic acid and ethyl 6-bromo-3-chloro-2-naphthalenecarboxylate [palladium(triphenylphosphine)$_4$ (Aldrich, St. Louis, MO), aqNa_2CO_3, dimethoxyethane, reflux, 6 h], followed by chromatography (6% EtOAc/hexane on silica gel) to give the benzyl-protected ethyl ester of MM11453 (66%). Benzyl group cleavage [BBr_3, CH_2Cl_2, −78°C, 2 h] to the phenol (91%) and ester group hydrolysis (aq NaOH, ethanol, 90°C, 2 h; aq HCl) gave MM11453 (95%) as a white powder, melting point 294°C–296°C (decomp.). IR (KBr): 3200, 1706, 1277, 1244, 991, 815, and 680 cm^{-1}. ^1H nuclear magnetic resonance (300 MHz, Me_2SO-d_6, δ): 1.75, 2.06, 2.17 (s, 6, adamantyl CH_2; s, 3, adamantyl CH;

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[3] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid; aq, aqueous; GST, glutathione *S*-transferase; *tk*, thymidine kinase; CAT, chloramphenicol acetyltransferase; DPSA, differential protease sensitivity assay; TRE, thyroid hormone receptor response element; TREpal, palindromic TRE; PF, protease-resistant fragment; Hsp, heat shock protein; LBD, ligand-binding domain; Met, methionine; NCoR, nuclear receptor corepressor; Rh123, rhodamine green; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid.

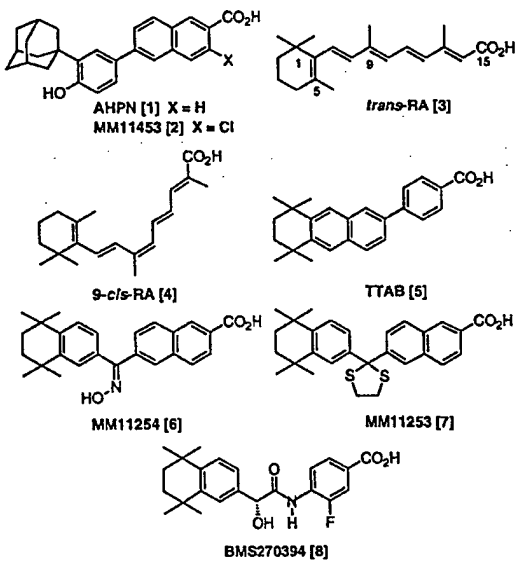

Fig. 1. AHPN [1], MM11453 [2], *trans*-RA [3], 9-*cis*-RA [4], TTAB [5], MM11254 [6], MM11253 [7], and BMS270394 [8].

s, 6, adamantyl CH_2), 6.97 (d, J = 9.0 Hz, 1, ArH-5), 7.51 (s, 1, ArH-2), 7.52 (d, J = 9.0 Hz, 1, ArH-6), 7.98 (d, J = 9.0 Hz, 1, NapH-8), 8.06 (s, 1, NapH-5), 8.25 (d, J = 8.6 Hz, 1, NapH-7), 8.27 (s, 1, NapH-4), 8.60 (s, 1, NapH-1), 9.68 (s, 1, ArOH). High-resolution mass spectrometry for $C_{27}H_{25}ClO_3$ (M^+): calculated, 432.1492; found, 432.1492. *trans*-RA [3] was purchased (Sigma Chemical Co.), as was [11,12-$^3H)_2$]9-*cis*-RA (specific activity, 43 Ci/mmol; DuPont NEN, Boston, MA). 9-*cis*-RA [4] was prepared as reported (29).

Computational Analysis. CAChe Software (Fujitsu, Beaverton, OR) was used to identify low-energy conformers within 2 kcal of the global energy minimum (MM3 force field, conjugate-gradient minimization, 30° search label variation, exclusion of ≥9 Å van der Waals interactions, and energy change <0.001 kcal/mol). Conformers were superimposed by using least-squares rigid fit of atoms corresponding to the 1, 5–9, and 15 carbon molecules of *trans*-RA.

Receptor Transcriptional Activation.[4] CV-1 cells (1,000 per well) were grown in DMEM (Irving Scientific, Santa Ana, CA) with 10% charcoal-treated FCS (Tissue Culture Biologicals, Tulare, CA) for 16–24 h before transfection, as described (30, 31). Briefly, 100 ng of $(TREpal)_2$-*tk*-CAT reporter, β-galactosidase expression vector pCH 110 (Pharmacia, Piscataway, NJ), and a RAR expression vector (or 20 ng of RXRα) were mixed with carrier DNA (pBluescript; Stratagene, La Jolla, CA) to give 1 μg of total DNA/well. CAT activity was normalized using β-galactosidase as the control. Activation after subtraction of constitutive activity is expressed relative to that of 1.0 μM *trans*-RA for RARs (100%) or 1.0 μM 9-*cis*-RA for RXRα (100%) and represents the average of three determinations.

Receptor Binding. Competitive radioligand binding on crude bacterial lysates at 0°C for 2 h used ~25 μmol of recombinant human RAR subtype or mouse RXRα-GST fusion proteins in 200 μl of binding buffer [10 mM HEPES (Sigma Chemical Co.; pH 7.8), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 10% glycerol] with 1–2 nM [$^3H_2$]9-*cis*-RA (43 Ci/mmol). Bound [^{3}H]9-*cis*-RA was isolated (Sephadex G-50; Pharmacia) and counted. Nonspecific [^{3}H]9-*cis*-RA binding at 1 μM nonlabeled 9-*cis*-RA generally was <10% of total label bound.

DPSA. [^{35}S]Met-labeled RARα, RARβ, RARγ, and RXRα, prepared by *in vitro* transcription/translation (32), were used in DPSA as described (33). [^{35}S]Met-labeled receptors were incubated with 0.1% ethanol alone, 1.0 μM 9-*cis*-RA, or MM11453 for 30 min at 0°C. Limited proteolysis (trypsin-tosyl phenylalanyl chloromethyl ketone; Sigma Chemical Co.) for 15 min at 22°C, followed by termination by Laemmli sample buffer and boiling and separation (10% acrylamide gel under denaturing conditions), afforded PFs for visualization by autoradiography (33, 34).

GST-Pulldown. Experiments were performed as described using GST-p300 1–450 (35) and GST-NCoR 2110–2453 (36) fusion proteins and [^{35}S]Met-labeled human RARγ.

Cell Lines and Culture. RA-resistant HL-60R cells, having a mutant RARα that does not significantly bind *trans*-RA and lacking RARβ and RARγ (26), and MDA-MB-231 cells were grown as described (20). MCF-7, LNCaP prostate, H460 and retinoid-resistant H292 lung cancer cells and Jurkat lymphoma cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Irving Scientific) with 10% charcoal-treated FCS.

Cell Growth Inhibition. HL-60R and MDA-MB-231 cells (50,000 and 100,000 per well, respectively) and 0.1–1.0 μM MM11453, AHPN, or Me_2SO alone were incubated for 24 or 120 h (72-h medium change), respectively. Results are expressed relative to Me_2SO control as mean ± SE of triplicate experiments. SEs were <10%. MCF-7, LNCaP, H292, and H460 cells (3,000 per well in 96-well plates) were treated with 1.0 μM MM11453, AHPN, *trans*-RA, or ethanol alone for 48 h before viable cell numbers were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (7, 9, 27). Data shown are representative of three experiments.

Apoptosis Detection. DNA fragmentation and apoptotic bodies were assessed in at least 500 HL-60R or MDA-MB-231 cells after incubation with MM11453 for 24 or 120 h, respectively, as described above, and acridine orange staining (15). The percentage of apoptotic cells was expressed relative to the Me_2SO control as the mean ± SE of triplicate experiments. MCF-7, LNCaP, H292, H460, and Jurkat cells (3,000 per well) were treated with 1.0 μM MM11453, *trans*-RA, or ethanol alone for 48 h, trypsinized, washed (PBS), fixed (3.7% paraformaldehyde), and stained with 4',6-diamidino-2-phenylindole (1 μg/ml) to visualize nuclei by fluorescent microscopy (21). Cells with apoptotic nuclear morphology were scored in each 400-cell sample using a fluorescence microscope. The data are representative of three experiments.

Northern Analysis. Total RNAs were prepared (RNeasy Mini kit; Qiagen, Germany), and TR3 expression was determined on 30 μg of total RNA from each line treated with 1.0 μM MM11453, *trans*-RA, or ethanol alone. Blotting conditions were as described (27) with β-actin expression as the control.

TR3 Mitochondrial Targeting. The expression vector for TR3/ΔDBD-GFP, a TR3 mutant lacking the DNA-binding domain fused to the green fluorescent protein expression vector, was transiently transfected into H460 cells, as described for LNCaP cells (27). Cells were treated with 1.0 μM MM11453 or ethanol alone for 6 h and then immunostained with anti-Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Cy3-conjugated secondary antibody (Sigma Chemical Co.) to indicate mitochondria to which Hsp60 is restricted. Confocal microscopy was used to detect TR3/ΔDBD-GFP (green fluorescence) and Hsp60 (red). Images were overlaid to show colocalization.

Mitochondrial Membrane Potential Assay. LNCaP, MCF-7, and MDA-MB-231 cells (10,000,000) were treated with 1.0 μM MM11453 for 18 h before incubation with 5 μg/ml Rh123 for 30 min at 37°C. Rh123-fluorescing cells were scored depolarized by flow cytometry (FACScalibur system; BD Biosciences, San Jose, CA; Ref. 37). The data shown are representative of three experiments. Wild-type Jurkat cells or Jurkat cells stably expressing either Bcl-2 or control vector (38) were treated similarly.

RESULTS

Close-Fitting of Energy-minimized AHPN and Retinoid Conformers. Energy-minimized conformers of AHPN [1], RAR-selective *trans*-RA [3], and RAR-selective TTAB [5] (39) were overlapped. The *trans*-RA conformer was that reported in the RARγ LBD (40, 41). Three orthogonal views of these overlaps are shown in Fig. 2*A*. The major structural difference was the 1-adamantyl group of AHPN, which extended 2.2 Å more than the *trans*-RA 18-methyl group. In Fig. 2*B*, the energy-minimized conformers of RARγ-selective agonists AHPN and MM11254 [6], a (Z)-oxime (14) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid (42), are shown overlapped with RARγ-selective agonist BMS270394 [8], as found in the ligand-binding pocket of crystallized holo-RARγ (40). The

[4] Assay conducted at The Burnham Institute, La Jolla, CA, under a license agreement with Ligand Pharmaceuticals, San Diego, CA.

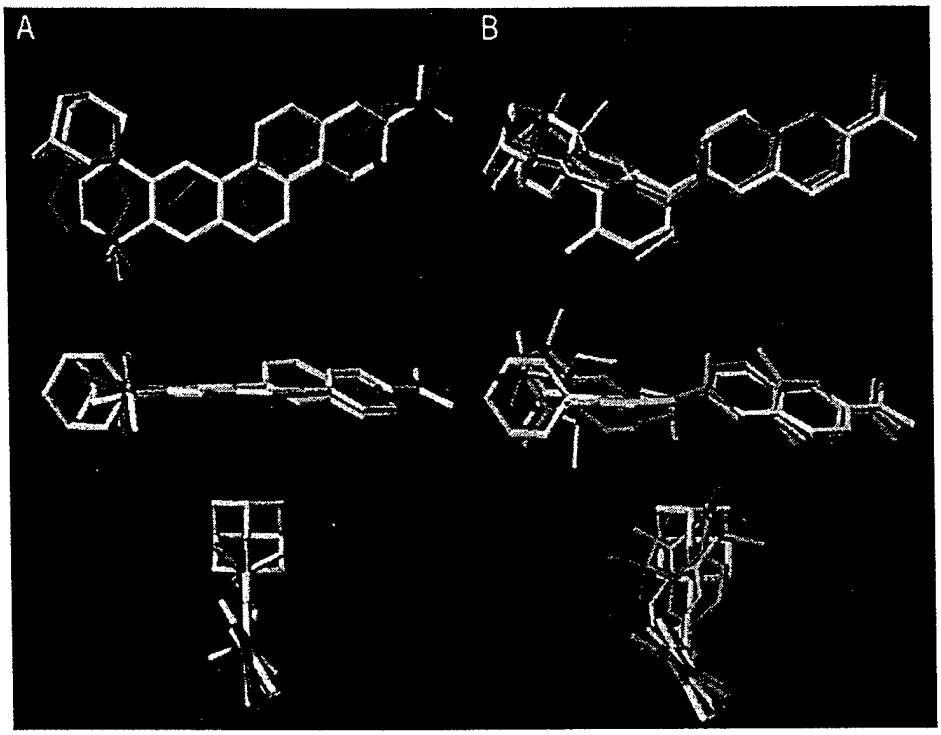

Fig. 2. Comparison of energy-minimized AHPN and retinoid conformers. Conformational analysis was performed as described in "Materials and Methods." *A*, orthogonal views of superimposed conformers of AHPN (*blue*), *trans*-RA (*red*), and TTAB (*yellow*). *B*, superimposed conformers of AHPN (*blue*), MM11254 (*green*), and BMS270394 (*magenta*).

AHPN 1-adamantyl group overlaps the saturated portion of the 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene rings of both RARγ-selective retinoids, and the AHPN phenolic oxygen is near the oxygen molecules in the oxime group of MM11254 (2.5 Å) and the bridge hydroxyl of BSM270394 (3.9 Å). Such hydroxyl groups are reported to confer RARγ selectivity by hydrogen bonding to the Met-272 sulfur molecule of RARγ (41). Placement of these overlapped conformers (Fig. 2*B*) in the RARγ ligand-binding site gives ligand O–Met-272–S distances of 4.09, 2.55, and 3.32 Å, respectively. These studies suggest that binding of AHPN to RARγ occurs in the same manner as that of standard retinoid agonists.

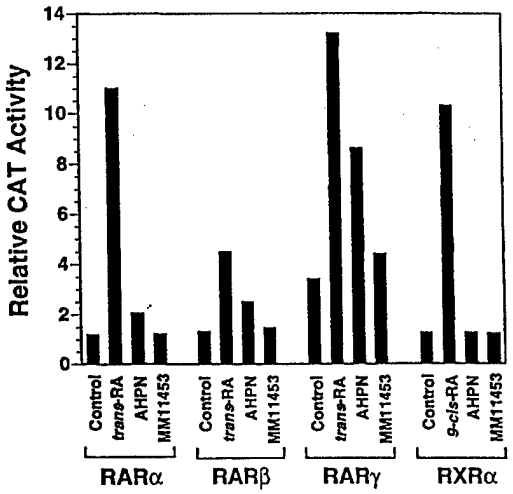

Fig. 3. Transcriptional activation of retinoid receptors by MM11453 on the $(TREpal)_2$-*tk*-CAT reporter. CV-1 cells were transiently transfected as described in "Materials and Methods," treated with 1.0 μM MM11453, AHPN, *trans*-RA, or 9-*cis*-RA, and assayed for CAT activity after 24 h. Reporter gene activation is expressed relative to 1.0 μM *trans*-RA on the RARs or 1.0 μM 9-*cis*-RA on RXRα.

MM11453 Lacked RAR Transcriptional Activation of AHPN. Although originally reported as RARγ selective on the $(TREpal)_2$-*tk*-CAT reporter in cotransfected HeLa cells (12), we observed on the $(TREpal)_2$-*tk*-CAT in CV-1 cells (4) that high RARγ selectivity occurred at 0.1 μM and below (14). At 0.5–1.0 μM, at which the natural retinoid *trans*-RA [3] inhibits retinoid-sensitive cancer cells, AHPN significantly activated RARβ. At 1.0 μM AHPN, reporter activation by RARα, RARβ, and RARγ was 9, 37, and 54%, respectively, of that caused by 1.0 μM *trans*-RA (Fig. 3). Unlike 1.0 μM *trans*-RA or 9-*cis*-RA, 1.0 μM MM11453 did not adequately activate any RAR subtype or RXRα to induce even modest $(TREpal)_2$-*tk*-CAT transcription. MM11453 did not activate RARα or RXRα and only activated RARβ and RARγ to 5 and 10%, respectively, of that of *trans*-RA or 13 and 19%, respectively, of that of AHPN. Thus, MM11453 is an analogue with substantially reduced capacity for RAR activation.

Retinoid Receptors Bound MM11453. Competitive ligand binding was used to determine whether MM11453 bound directly to RARs and RXRα. MM11453 at 1.0 μM displaced 61 ± 6% of $[^3H_2]$9-*cis*-RA bound to RARγ, whereas displacement from other receptors was lower [RARα (11 ± 2%), RARβ (25 ± 5%), and RXRα (18 ± 5%); Fig. 4].

MM11453 Did Not Induce an Agonist-bound RAR Conformation. DPSA on 9-*cis*-RA-bound RARα, RARβ, and RARγ produced 27-kDa PF27α, 35-kDa PF35β, and 32-kDa PF32γ, respectively (*Lane 3* in Fig. 5, *A–C*). DPSA on AHPN-bound RARs produced the same fragments (data not shown). These PFs were not observed on incubation with ethanol or MM11453 (*Lanes 2* and *4*, respectively, in Fig. 5, *A–C*). Unlike 9-*cis*-RA, neither MM11453 nor AHPN altered the proteolytic sensitivity of RXRα (data not shown). The lack of PFs from RAR-MM11453 complexes suggests that MM11453 does not

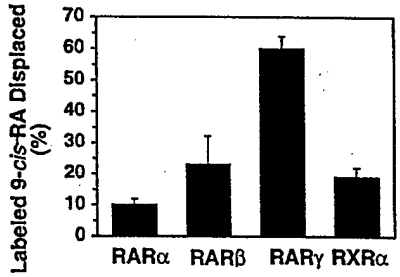

Fig. 4. Binding affinity of MM11453 to recombinant RAR and RXRα. Competition radioligand binding was conducted as described in "Materials and Methods." The data represent the means (n = 3) of the percentages of [11,12-$^3H_2$]9-*cis*-RA bound that were inhibited by 1.0 μM MM11453; *bars*, SE.

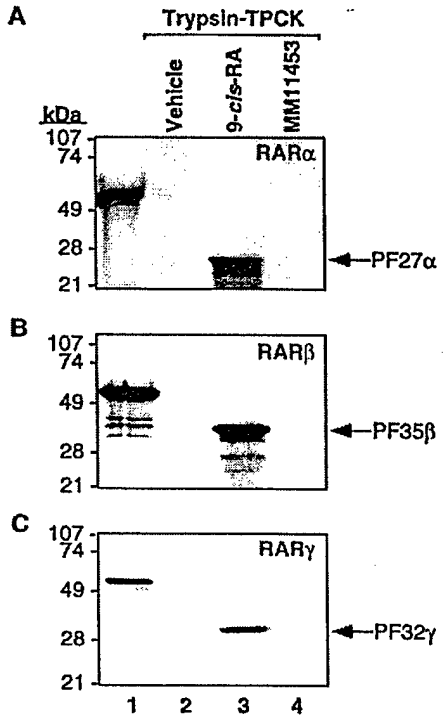

Fig. 5. MM11453 is not a RAR agonist. *A*, DPSA on [^{35}S]Met-labeled RARα in ethanol or 1 μM 9-*cis*-RA or MM11453. The migration of the 9-*cis*-RA-induced PF27α of RARα is indicated. In *B* and *C*, DPSA on RARβ and RARγ, respectively, were conducted as in *A* and "Materials and Methods." Positions of RARβ PF35β and RARγ PF35β are indicated. *Left*, marker migration (molecular mass).

promote an agonist-bound conformation. Similar to RARγ-selective antagonist MM11253,[5] MM11453 did not prevent RAR/RXR agonist 9-*cis*-RA from inducing this conformation in RARα, RARβ, or RARγ (data not shown).

MM11453 Failed to Dissociate Corepressor NCoR-RARγ *in Vitro*. GST-pulldown was used to test whether MM11453 dissociated NCoR (5) from RARγ, as 9-*cis*-RA does. As indicated (Fig. 6*A*), 9-*cis*-RA (*Lane 3*), but not MM11453 (*Lane 4*) or vehicle (*Lane 2*), disrupted the NCoR-RARγ complex.

MM11453 Failed to Recruit Coactivator p300 to RARγ. We compared the abilities of MM11453 and 9-*cis*-RA (36) to recruit p300 (43) to RARγ. Vehicle (*Lane 2* in Fig. 6*B*) or MM11453 (*Lane 4*) did not enhance p300 recruitment, whereas 9-*cis*-RA did (*Lane 3*). These findings, which agree with results on MM11453 in RARγ DPSAs (Fig. 5) and corepressor-dissociation experiments (Fig. 6*A*), confirm that MM11453 does not induce a RARγ-agonist conformation.

MM11453 Inhibited Cancer Cell Growth. Increasing evidence, including retinoid-resistant cancer cell growth inhibition (13, 27), suggests that AHPN action is independent of retinoid receptors (21, 44). Cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were conducted to show that MM11453 inhibited growth similarly. MM11453 inhibited HL-60R and MDA-MB-231 growth with IC_{50}s of 0.17 and 0.32 μM (Fig. 7, *A* and *B*), respectively, compared with AHPN values of 0.15 and 0.30 μM, respectively. Inhibition by 1.0 μM *trans*-RA was ≤5% (13). The effects of MM11453 on H460, H292, LNCaP, and Jurkat cells were then examined. As shown (Fig. 7*C*), 1.0 μM MM11453 significantly reduced growth by 70, 46, 64, and 70%, respectively, whereas 1.0 μM *trans*-RA reduced H460 growth by 15% and had no evident effect on the other lines (0–3%). Some of us reported previously that 1.0 μM AHPN for 48 h inhibited the growth of H460, H292, and LNCaP cells by 62 ± 6% (21), 53 ± 5% (21), and 100 ± 5% (25), respectively, whereas Jurkat growth was inhibited by 84% and 80 ± 3% after 24 and 96 h, respectively (22). Thus, both AHPN and MM11453 similarly retard the growth of these cell lines.

MM11453 Induced Cancer Cell Apoptosis. The MM11453 EC_{50}s for inducing nuclear fragmentation in HL-60R and MDA-MB-231 cells were 0.12 and 0.13 μM, respectively (Fig. 7, *D* and *E*), which are similar to AHPN EC_{50}s of 0.07 and 0.35 μM, respectively (13). HL-60R apoptosis inhibition by 1.0 μM MM11453 and AHPN was 82 ± 3% and 91 ± 4%, respectively, and MDA-MB-231 apoptosis

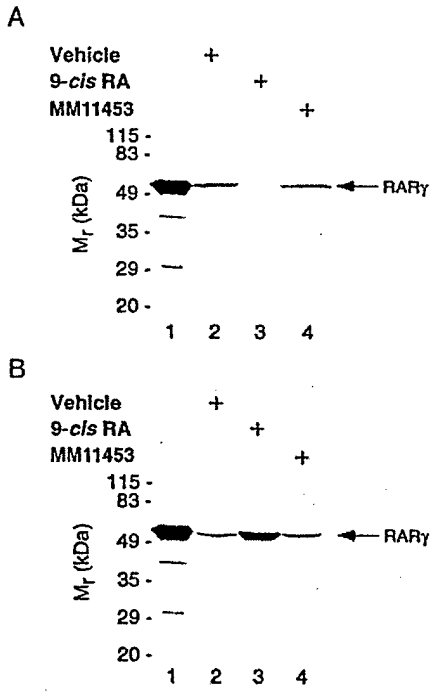

Fig. 6. MM11453 does not induce NCoR corepressor dissociation from RARγ or coactivator p300 recruitment to RARγ. *A*, NCoR-RARγ dissociation using [^{35}S]Met-RARγ and GST-NCoR 2110–2453 is as described in "Materials and Methods." Only 9-*cis*-RA induced NCoR-RARγ dissociation (*Lane 3*). In *B*, RARγ coactivator recruitment using GST-p300 (1–450) and [^{35}S]Met-RARγ is as in "Materials and Methods." Only 9-*cis*-RA enhanced binding of p300 to RARγ. *Lane 1* in *A* and *B* represents ~15% of [^{35}S]Met-RARγ. *Left*, marker migration (molecular mass).

[5] V. J. Peterson, M. I. Deinzer, M. I. Dawson, K-C. Feng, A. Fields, and M. Leid. Mass spectrometric analysis of agonist-induced retinoic acid receptor γ conformational change, unpublished results.

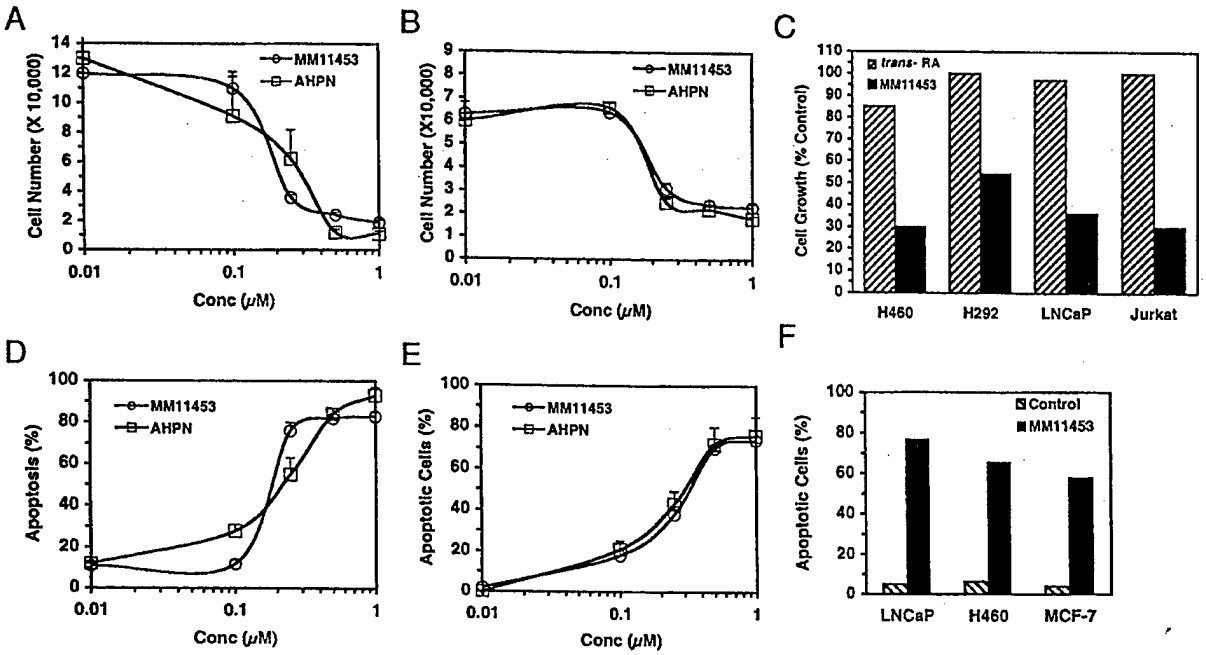

Fig. 7. MM11453 inhibits cell growth and induces apoptosis. HL-60R cells (*A* and *D*) and MDA-MB-231 cells (*B* and *E*) were treated with 10 nM to 1.0 μM MM11453, AHPN, or Me_2SO alone for 24 h and 120 h, respectively, as described in "Materials and Methods," and then harvested and counted (*A* and *B*) or assayed for apoptosis (*D* and *E*) as in "Materials and Methods." The results shown represent the means of three replicates; *bars*, SE. In *C* and *F*, H460, H292, LNCaP, and Jurkat cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 48 h before viability was determined (*C*) or treated with 1.0 μM MM11453 or ethanol alone for 48 h before nuclear morphology was analyzed (*F*) as in "Materials and Methods." The experiments shown are representative of three triplicates.

was 75 ± 1% and 76 ± 7%, respectively. Thus, both MM11453 and AHPN are similarly apoptotic in retinoid-resistant cells. MM11453 at 1.0 μM induced apoptosis in LNCaP (38%), H460 (47%), and MCF-7 (57%) cells, as demonstrated by nuclear morphological changes (Fig. 7*F*). In other experiments using these cells, 1.0 μM AHPN was found to induce 21 (21), 37, and 42% apoptosis, respectively (data not shown). Thus, both MM11453 and AHPN also induce apoptosis in retinoid-sensitive cells.

MM11453 Induced TR3 Expression. TR3 expression must be induced for AHPN to cause lung cancer cell apoptosis (21). To determine whether 1.0 μM MM11453 had this capability, H460 and LNCaP cells were treated for 6 h. MM11453 strongly induced TR3 expression, whereas *trans*-RA did not (Fig. 8).

MM11453 Induced TR3 Mitochondrial Targeting. MM11453 at 1.0 μM induced the migration of transiently expressed TR3/ΔDBD-GFP to mitochondria in H460 cells, as indicated in Fig. 9 by colocalization of GFP fluorescence with that of immunostained Hsp60. Colocalization did not occur in vehicle-alone-treated cells (data not shown). Thus, both AHPN and MM11453 induce TR3 targeting to mitochondria.

MM11453 Altered Mitochondrial Membrane Potential. We found that MM11453 induced TR3 targeting to the mitochondrial outer membrane of breast and prostate cancer cells to initiate cytochrome *c* release and apoptosis (27). A loss of inner mitochondrial membrane potential or depolarization, which may signify outer membrane or permeability transition pore opening (45) and has been suggested as causing cytochrome *c* release (45), is associated with apoptosis. The effect of MM11453 on this process was explored using Rh123, which cells incorporate on depolarization. MM11453 increased MCF-7, MDA-MB-231, and LNCaP cell Rh123 fluorescence 2.2-, 1.9-, and 5.4-fold, respectively (Fig. 10). Again, MM11453 behaves similarly to AHPN (46).

Bcl-2 Attenuated Mitochondrial Membrane Depolarization by MM11453. Because overexpression of antiapoptotic, mitochondrial membrane-surface protein Bcl-2 is reported to block cancer cell apoptosis (47), its effect on apoptosis by MM11453 was explored in Jurkat cells transfected with an expression vector containing *bcl-2* or the vector alone. In MM11453-treated nontransfected cells and MM11453-treated vector alone-transfected cells, depolarized cell numbers increased 4.8- and 5.5-fold, respectively, over that of the nontreated control, whereas cell numbers increased only 2-fold in treated cells overexpressing *bcl-2* (Fig. 11). Thus, *bcl-2* modified the effect of MM11453 on mitochondrial membranes.

DISCUSSION

AHPN induces apoptosis in cancer cell lines (13, 14, 18–22, 24). How AHPN initiates this process remains to be completely defined. A report of the RARγ selectivity of AHPN (12) led to the hypothesis of an apoptotic role for RARγ in breast cancer, melanoma, and neuroblastoma cells (17, 23, 24). To support this, RARγ transcriptionally active AHPN derivatives and analogues were also reported to inhibit growth and induce apoptosis (28, 48, 49). Other reports present data strongly suggesting an RAR-independent pathway, such as growth inhibition and apoptosis of retinoid-resistant cancer cells (13, 14, 21, 27, 42, 44, 50).[5] Our results support the latter by showing that MM11453, although unable to activate retinoid receptors on a reporter with the efficacy of standard retinoids or AHPN, strongly inhibited growth and induced apoptosis in retinoid-resistant cancer cell lines.

The near absence of RAR subtype and RXRα transcriptional activation by MM11453 was confirmed by limited proteolysis. DPSAs suggest that MM11453 is not a RAR or RXRα agonist. MM11453 did not induce a protease-resistant RARγ conformation, characteristic of binding a retinoid agonist, such as MM11254 [6], but behaved as the

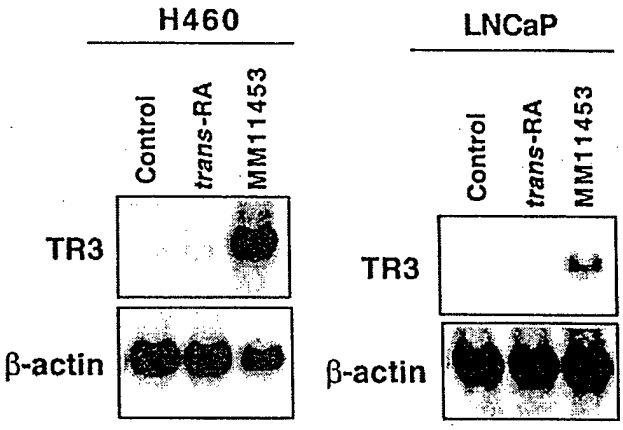

Fig. 8. MM11453 induces TR3 expression in H460 and LNCaP cells. Cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 6 h. Total RNAs were prepared and analyzed for TR3 expression by Northern blotting. Expression of β-actin was the RNA-loading control.

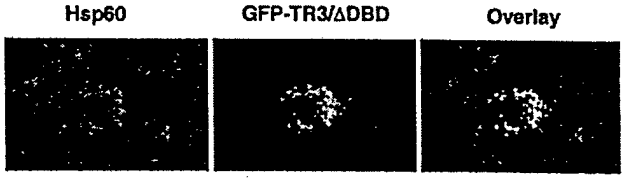

Fig. 9. MM11453 induces TR3 translocation to H460 mitochondria. Cells were transiently transfected with TR3/ΔDBD-GFP expression vector and then treated with 1.0 μM MM11453 for 6 h as in "Materials and Methods." Immunostained mitochondrial Hsp60 (*red*) and TR3/ΔDBD-GFP protein (*green*) were visualized by confocal microscopy, and images were overlaid (*Overlay*) to indicate colocalization (*yellow*).

RARγ-selective antagonist MM11253 [7], a dithiane (14, 39) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid].[5] MM11453 did not detectably dissociate NCoR from RARγ or recruit p300 to RARγ, as agonists did. Thus, the behavior of MM11453 contrasts with that of RAR-agonist AHPN (12, 14). A retinoid receptor-independent pathway for anticancer activity has precedent in the mechanism of action of *N*-(4-hydroxy)phenyl retinamide, which inhibits the growth of cancer cells that resist standard retinoids (51, 52).

DPSA (data not shown) and molecular modeling (Fig. 2) suggest that AHPN does not induce a unique conformation in the RARγ LBD that could account for apoptosis-inducing activity. RARγ on binding AHPN, *trans*-RA, or MM11254 produced the same PFs,[5] whereas 1.0 μM MM11253 [7] did not induce this conformation[5] or transcriptionally activate RARγ (14). Both transactivation and DPSA show that the bulky 1-adamantyl group of AHPN (Fig. 2*A*) did not prevent an agonist-induced RARγ conformation, and modeling shows the 1-adamantyl group occupying the same region as the tetrahydronaphthalene rings of agonists MM11254 and BMS270394 [8] (Ref. 41; Fig. 2*B*). The three hydroxyl and carboxyl oxygen molecules are also close. Thus, on the basis of the strategy used by Klaholz *et al.* (41) that the low-energy conformation of a ligand approximates its bound form, our findings suggest that pharmacophoric AHPN groups are not responsible for inducing any unique conformation in RARγ. Only the 3-chloro group *ortho* to the COOH group distinguishes MM11453 from AHPN. How the chloro group inhibits transcriptional activation remains to be determined. Both its steric and electronic properties may perturb hydrogen bonding by the COOH group or shift van der Waals contacts of RARγ LBD pendant groups, thereby preventing the conformational changes in the receptor necessary for coactivator recruitment and transcriptional activation.

The inhibition of [^{3}H]9-*cis*-RA binding to RARs by MM11453 suggests direct binding, whereas transfection indicates minimal RAR or RXRα agonism. Thus, MM11453 may function as a moderately selective RARγ antagonist. Although how RARγ antagonism or that of another RAR or RXR subtype contributes to MM11453 activity remains to be defined, the lack of growth inhibition by antagonist MM11253 (data not shown) suggests that the contribution, if any, is small. Unlike *trans*-RA, both MM11453 and AHPN strongly inhibited HL-60R, MDA-MB-231, LNCaP, and H292 cell growth and induced apoptosis. EC_{50}s for inhibiting growth in HL-60R and MDA-MB-231 cells were comparable, and their apoptotic EC_{50}s were similar (Fig. 7). These results indicate that MM11453 functions independently of RARs and RXRα and strongly suggest a similar mode of action for AHPN. Both AHPN (21) and MM11453 (Fig. 8) induced TR3 expression in H460 and LNCaP cells and TR3 mitochondrial translocation (Ref. 27 and Fig. 9, respectively) and caused inner mitochondrial membrane depolarization in MCF-7, MDA-MB-231, LNCaP, and Jurkat cells (Figs. 10 and 11). These results demonstrate that

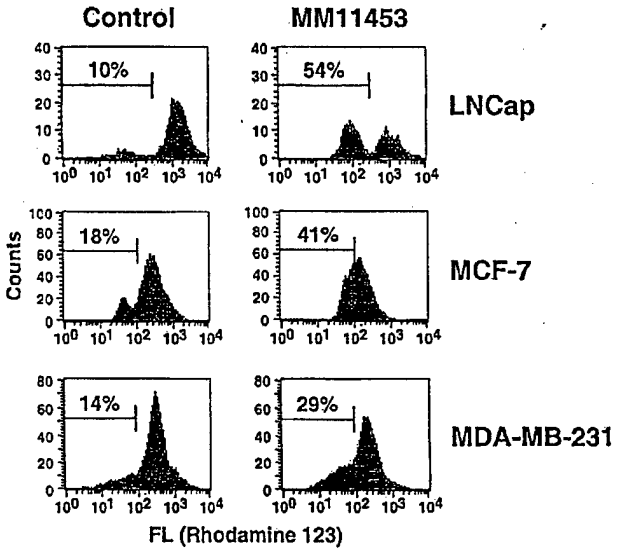

Fig. 10. Effect of MM11453 on LNCaP, MCF-7, and MDA-MB-231 mitochondrial membrane potential. Cells were treated with or without 1.0 μM MM11453 for 18 h and then with Rh123 as in "Materials and Methods." Rh123-fluorescencing cells are expressed as a percentage of the total.

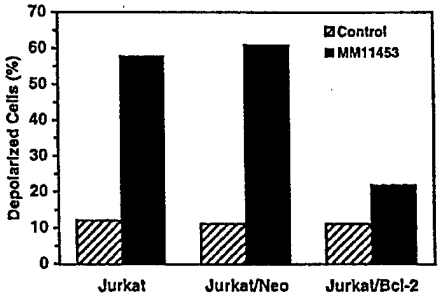

Fig. 11. Bcl-2 inhibits Jurkat mitochondrial membrane potential decrease by MM11453. Nontransfected cells stably expressing vector alone (*Jurkat/Neo*) and transfected cells stably expressing Bcl-2 (*Jurkat/Bcl-2*) were treated with 1.0 μM MM11453 or ethanol alone for 18 h and analyzed for change in mitochondrial membrane potential as in "Materials and Methods."

MM11453 retains the apoptotic properties of AHPN without behaving as a competent RARγ agonist and, thus, indicate that RARγ activation is not required for apoptotic activity. The recent report that AHPN induces apoptosis in RARγ-negative myeloma cells through a mitochondrial pathway (46) supports this conclusion. Reporter and limited proteolysis assays on MM11453 and AHPN suggest that their apoptotic activity does not involve RARα, RARβ, or RXRα activation.

Transactivation by liganded RARγ is reported to correlate with retinoid toxicity (53, 54). The lack of retinoid receptor activation activity by MM11453 suggests that toxic side effects characteristic of retinoid receptor activation (reviewed in Ref. 11) should be reduced in this class of apoptosis inducers, thereby affording more effective candidates for development as cancer chemotherapeutic agents.

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Vitamin A and apoptosis in prostate cancer

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Abstract

Apoptosis represents an effective way to eliminate cancer cells. Unfortunately, advanced prostate tumors eventually progress to androgen-independent tumors, which are resistant to current therapeutic approaches that act by triggering apoptosis. Vitamin A and its natural and synthetic analogs (retinoids) induce apoptosis in prostate cancer cells *in vitro* and in animal models, mainly through induction of retinoic acid receptor-β (RARβ). Expression levels of RARβ, however, are significantly reduced in hormone-independent prostate cancer cells. Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) effectively induces apoptosis of both hormone-dependent and -independent prostate cancer cells in a retinoid receptor-independent manner. The apoptotic effect of AHPN requires expression of orphan receptor TR3 (also called nur77 or NGFI-B). Paradoxically, TR3 expression is also induced by androgen and other mitogenic agents in prostate cancer cells to confer their proliferation. The recent finding that TR3 migrates from the nucleus to mitochondria to trigger apoptosis in response to AHPN suggests that the opposing biological activities of TR3 are regulated by its subcellular localization. Thus, agents that induce translocalization of TR3 from the nucleus to mitochondria will have improved efficacy against prostate cancer. TR3, therefore, represents an unexplored molecule that may be an ideal target for developing new agents for prostate cancer therapy.

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Introduction

Prostate cancer is the most common cancer diagnosed among men in the United States, accounting for 27.5% of all cancer cases in men. It ranks second after lung cancer as the underlying cause of cancer death in US men. Despite aggressive efforts toward earlier detection and treatment, the mortality rate for prostatic carcinoma has steadily increased. The identification of androgens as the major regulator of prostatic epithelial proliferation offered a target for therapeutic intervention. Androgen ablation by surgical gonadectomy or drug treatments that suppress androgen production and action remain the only effective form of therapy for men with advanced disease. Unfortunately, the median duration of response to androgen ablation is less than 2 years, after which the disease will re-emerge in a poorly differentiated, androgen-independent form, which is often fatal. The lack of therapies for this advanced prostate cancer has contributed significantly to the increased mortality rates, and has resulted in the impetus to develop non-androgen-based therapies.

Vitamin A and its natural and synthetic analogs, retinoids, are one of the most investigated classes of chemopreventive drugs for prostate cancer. Early experiments on mouse prostate explant cultures showed that all-*trans*-retinoic acid (*trans*-RA) could both inhibit and reverse the proliferative effects of chemical carcinogens on prostatic epithelium (Lasnitzki & Goodman 1974, Chopra & Wilkoff 1976). Recent studies have demonstrated that retinoids effectively inhibit the growth of prostate cancer cells *in vitro* and suppress the development of prostate carcinogenesis (Blutt *et al.* 1997, DiPaola *et al.* 1997, Campbell *et al.* 1998, Goossens *et al.* 1999, McCormick *et al.* 1999, Pasquali *et al.* 1999, Richter *et al.* 1999, Sun *et al.* 1999*b*, Urban *et al.* 1999, Webber *et al.* 1999, Kelly *et al.* 2000, Koshiuka *et al.* 2000, Lotan *et al.* 2000, Tanabe 2000, Pili *et al.* 2001). Clinical trials of several retinoids and their combination with other anti-cancer agents have shown significant activities when retinoids were used in combination with other chemotherapeutic agents, such as interferon-α and paclitaxel (DiPaola *et al.* 1997, 1999, Culine *et al.* 1999, Shalev *et al.* 2000, Thaller *et al.* 2000). Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) (Bernard *et al.* 1992) was found to potently inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate cancer cells. Thus, these small molecules may serve as prototypes for the

development of new prostate cancer therapeutic and preventive agents. The recent identification of the molecular targets of retinoid action in prostate cancer cells offers opportunities for the development of novel therapeutic strategies.

Vitamin A signaling pathways

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). RARs and RXRs are encoded by three distinct genes (α, β and γ). In addition, many retinoid receptor isoforms are generated through differential promoter usage, giving rise to a large number of distinct retinoid receptor proteins. To date, there are dozens of receptors which are known to mediate the effect of retinoids cloned. 9-*cis* RA is a high-affinity ligand for both RARs and RXRs, whereas *trans*-RA is a ligand for only RARs. Retinoid receptors belong to a large steroid/thyroid receptor superfamily that mediate the biological effects of many hormones, vitamins and drugs. RARs and RXR act as transcriptional factors to positively or negatively regulate expression of target genes by binding to their response elements (RAREs) located in promoter regions of the target genes (Fig. 1). The physiological role of RARs and RXRs has been extensively studied by knockout experiments (Kastner *et al.* 1995). Knockout of most of individual RARs activity by homologous recombination appears normal due to redundancy in the function of RARs *in vivo*. However, knockout of RARα and RARγ as well as RAR double knockouts produces defects that resemble the postnatal vitamin A-deficient syndrome and can be prevented by *trans*-RA administration, including keratinizing squamous metaplasia of the prostate gland (Kastner *et al.* 1995).

RXRs form heterodimers with many nuclear receptors including RARs, thyroid hormone receptor (TR), vitamin D receptor and peroxisome proliferator-activated receptor (PPAR) (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995), thereby mediating diverse endocrine signaling pathways. The function of RARs, however, is more restricted. The role of ligands in the regulation of retinoid receptor function is complex. RAR/RXR is activated mainly through binding of RAR with its ligand, although there are some situations where binding of both the RAR and RXR components with their respective ligands can contribute to the activity of the RAR/RXR heterodimers (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). The retinoid binding to RXRs is required for the activation of RXR homodimers and certain RXR heterodimers, such as TR3/RXR and PPARγ/RXR (Zhang & Pfahl 1993, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). Unliganded retinoid receptors can act as negative transcription factors by binding to the RAREs of retinoid target genes, and recruit receptor corepressors, such as NcoR (Xu *et al.* 1999), leading to histone deacetylation and formation of an inactive chromatin structure preventing transcription. Binding of retinoids to their receptors induces receptor conformational changes that serve as switches by releasing the receptor corepressors and by facilitating the recruitment of receptor co-activators, such as CBP (Xu *et al.* 1999) (Fig. 1). Several of the co-activator proteins have histone acetylase activity that contributes to the formation of an active chromatin structure and results in the transcription of target genes.

In addition to their direct effects on transcription, liganded RAR can modulate the activity of other transcriptional factors, such as AP-1 (Pfahl 1993). Activated retinoid receptors can inhibit the activity of AP-1, thereby regulating the expression of AP-1 target genes. The inhibition of AP-1 activity is linked to the anti-proliferative effects of retinoids, and appears to be separable from their direct activation of transcription of retinoid-target genes. Synthetic retinoids that specifically inhibit AP-1 activity without activating transcription have been developed (Fanjul *et al.* 1994, Chen *et al.* 1995, Li *et al.* 1996). These AP-1-specific retinoids can inhibit cell proliferation *in vitro*.

Recent evidence indicating that the cytoplasmic action of several hormone receptors represents an important mechanism for regulating their biological function has accumulated. The proapoptotic effect of the orphan receptor TR3 (also known as nur77 and NGFI-B) does not require its transcriptional regulation because TR3 with its DNA-binding domain deleted is still capable of inducing apoptosis (Li *et al.* 2000). In contrast, the cytoplasmic action of TR3, through its mitochondrial targeting, is essential for its apoptotic activity (Li *et al.* 2000). The glucocorticoid receptor was also found to reside on mitochondria (Scheller *et al.* 2000), while differentiation of PC12 phaeochromocytoma cells is accompanied by nuclear export of NGFI-B (Katagiri *et al.* 2000). Estrogen receptors and androgen receptors trigger cell proliferation through their interaction with Src or phosphatidylinositol-3-OH kinase in the cytoplasm (Migliaccio *et al.* 2000, Simoncini *et al.* 2000, Kousteni 2001).

Apoptotic signalings

Apoptosis, also known as programmed cell death, is an evolutionarily conserved and indispensable process during normal embryonic development, tissue homeostasis and regulation of the immune system (Fisher 1994, Steller 1995, White 1996). The apoptotic process can be initiated by several different stimuli, including growth factor withdrawal, DNA damage, deregulation of the cell cycle or ligation of death receptors (Fisher 1994, Steller 1995, White 1996). These different apoptotic stimuli induce diverse early signaling events, which then converge by activating a common central biochemical pathway that is responsible for the execution of apoptosis. Execution of apoptosis is primarily mediated by caspases, a family of cysteine proteases with

The receptor may be divided into five regions (A, B, C, D, E, and F) based on structure and function similarities among members of the steroid / thyroid hormone receptor superfamily.

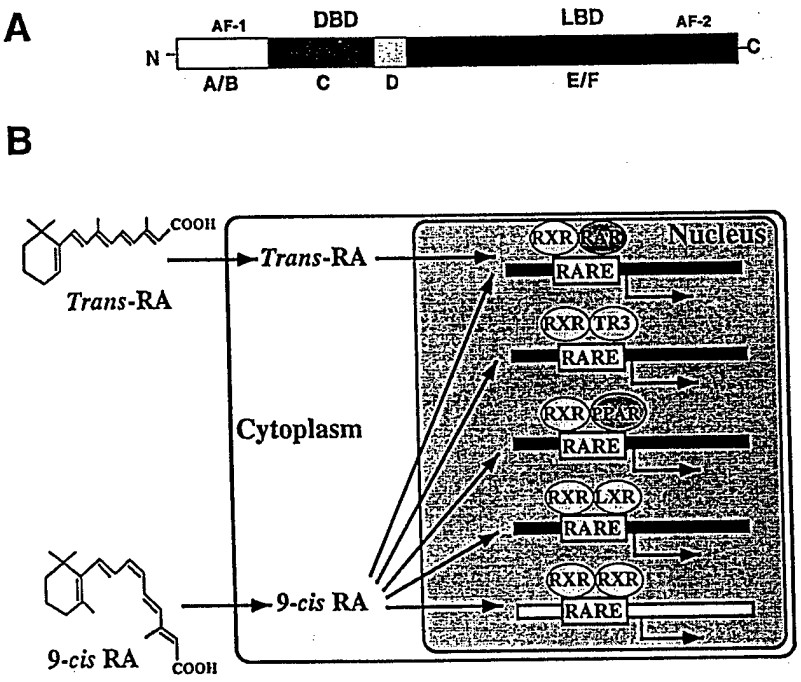

Figure 1 Retinoid signaling. Retinoid receptors are ligand-dependent transcription factors. (A) Schematic representation of retinoid receptor. DNA binding domain (DBD), ligand binding domain (LBD) and transactivation domains (AF-1 and AF-2) are indicated. (B) Mechanism of action of retinoid receptors. *Trans*-RA or 9-*cis* RA enter cells directly from the circulation, and bind to DNA-bound RAR or RXR, thereby eliciting a transcriptional response.

specificity for aspartic acid residues (Nunez *et al.* 1998, Thornberry & Lazebnik 1998).

There are two distinctly different pathways, the extrinsic and intrinsic pathways, transducing the death signals to caspase-mediated apoptotic machinery (Nunez *et al.* 1998). The extrinsic pathway involves activation of the superfamily of the tumor necrosis factor receptors (TNFR) or CD95 (Fas), by binding to their respective ligands, which in turn recruit procaspase-8 and -10 to membrane-associated signaling complexes, resulting in their activation (Fig. 2). Activation of these upstream caspases is sufficient to directly activate effector caspases such as caspase-3, -6 and -7, or indirectly induce apoptosis by cleaving Bid involved in the release of mitochondrial cytochrome c. The intrinsic pathway is activated directly by various forms of cellular stress that trigger mitochondrial release of cytochrome c into the cytosol. Cytosolic cytochrome c then binds to, and triggers oligomerization of the CED-4 homolog Apaf-1. The resulting 'apoptosome' recruits and activates procaspase-9 which, in turn, recruits and activates effector caspases, such as caspase-3 and possibly caspase-7 (Fig. 2). Additionally, the caspases can be activated by granzyme B, a major serine protease in cytotoxic lymphocyte granules (Shi *et al.* 1992). Once the effector caspases are activated, these enzymes cleave a number of cellular polypeptides leading to disassembly of key structural components of the nucleus and cytoskeleton, inhibition of DNA repair, replication, and transcription, and activation of endonucleases that irreversibly damage the genome (Fisher 1994, White 1996).

Members of the Bcl-2 family are known to modulate apoptosis in different cell types in response to various stimuli (Adams & Cory 1998, Reed 1998). Some members act as antiapoptotic proteins, such as Bcl-2 and Bcl-XL, whereas others function as proapoptotic proteins, such as BAX and BAK. Proapoptotic and antiapoptotic members can heterodimerize and seemingly titrate one another's function. Many Bcl-2 family proteins reside on the mitochondrial outer membrane (Adams & Cory 1998, Reed 1998). Bcl-2 prevents mitochondrial disruption and the release of cytochrome c from mitochondria, while Bax and Bak create pores in mitochondria membranes and induce cytochrome c release. In addition, most proapoptotic proteins antagonize antiapoptotic proteins through heterodimerization with them (Adams & Cory 1998, Reed 1998). Caspase-dependent apoptosis can also be regulated by members of the inhibitors of apoptosis (IAP) protein family. IAPs suppress apoptosis by physically interacting with and inhibiting the catalytic activity of caspases (Deveraux & Reed 1999). In apoptotic cells, the caspase inhibition by IAPs is negatively regulated by a mitochondrial protein Smac/DIABLO, which is released from the mitochondrial intermembrane space into the cytosol upon apoptotic stimuli (Du *et al.* 2000, Verhagen *et al.* 2000).

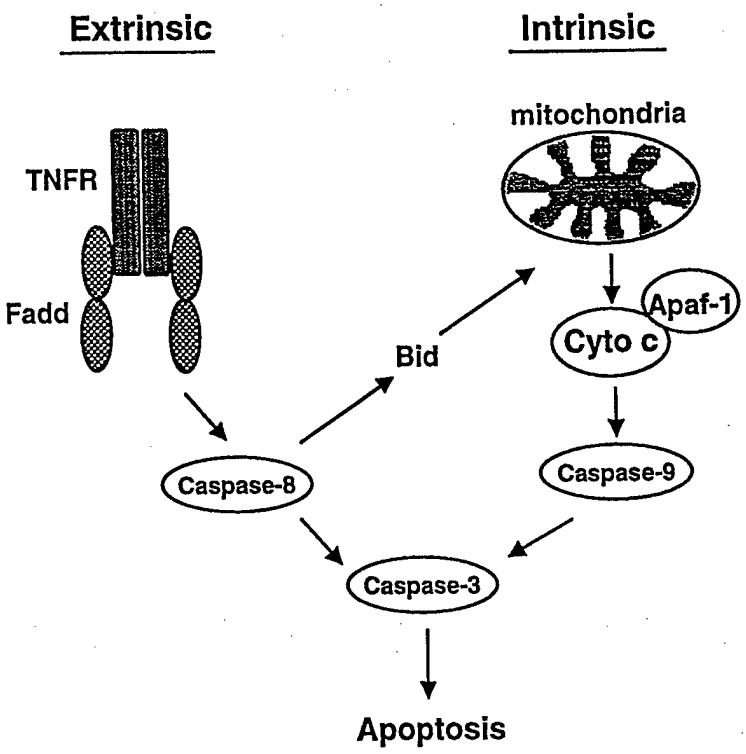

Figure 2 Apoptosis pathways. In the extrinsic pathway, ligation of death receptors activates initiator caspase-8 through the adaptor molecule Fadd. In the intrinsic pathway, cytochrome c (Cyto c) is released from mitochondria in response to a variety of death stimuli and binds to Apaf-1 to activate caspase-9. Active caspase -8 or -9 then activates effector caspases, such as caspase-3 resulting in morphological features of apoptosis. Caspase-8 also activates Bid, which then targets mitochondria to induce cytochrome c release, providing a link between the two pathways.

Apoptosis and prostate cancer development

Impaired apoptosis is involved in tumor initiation and progression, since apoptosis normally eliminates cells with increased malignant potential such as those with damaged DNA or aberrant cell cycling (Fisher 1994, Thompson 1995). Most prostate cancer cells have a protracted history of development, suggesting that prostate cancer cells must have evolved various mechanisms to subvert the apoptotic program (Bruckheimer & Kyprianou 2000). Impaired apoptosis signaling and extended cell survival seem to be closely associated with prostate tumor initiation, metastasis and progression to the androgen-insensitive state (Coffey *et al.* 2001). Increased levels of Bcl-2 are associated with emergence of an androgen-independent phenotype and overexpression of Bcl-2 can facilitate multistep prostate carcinogenesis in an animal model (Bruckheimer *et al.* 2000). Proapoptotic Bax contains a polymorphism in an unstable microsatellite causing a frameshift in androgen-independent DU145 cells (Rampino *et al.* 1997).

Recent studies have indicated a crucial role of the PTEN tumor suppressor in the regulation of prostate cancer development. PTEN catalyzes dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate and antagonizes signaling pathways that rely on PI3K activity (Wu *et al.* 1998). PTEN is frequently inactivated in primary human prostate cancers, particularly in the more advanced cancers (Ittmann 1998), in human prostate xenografts and in cell lines (Li *et al.* 1997, Vlietstra *et al.* 1998, Whang *et al.* 1998). Release of the negative regulation of the PI3K pathway by PTEN may activate the cell survival kinase Akt during prostate tumor progression (Stambolic *et al.* 1998). Indeed, activated Akt regulates a number of intracellular events implicated in prostate tumor progression and androgen independence. Disruption of PTEN leads to suppression of apoptosis (Stambolic *et al.* 1998), due to inactivation of Bad (Datta *et al.* 1997) or caspase-9 (Cardone *et al.* 1998) by Akt. The disruption can also accelerate cell cycle progression (Sun *et al.* 1999*a*), through suppression of AFX/Forkhead transcription factor activity by Akt (Brunet *et al.* 1999, Kops *et al.* 1999), resulting in inhibition of cell cycle inhibitor p27 expression (Medema *et al.* 2000). The central role played by PTEN has been recently confirmed by the finding that mice with double mutants

PTEN(+)/(–)p27(–)/(–) develop prostate cancer at complete penetrance within 3 months from birth (Di Cristofano *et al.* 2001).

Androgen ablation and apoptosis

Androgen withdrawal is the primary choice of therapy for men with advanced prostate cancer, and it generally leads to regression of the disease. It is believed that apoptosis is mainly responsible for the regression of prostate cancer cells (Buttyan *et al.* 2000) and increased levels of apoptosis were indeed observed in human prostate cancer cells after androgen withdrawal (Denmeade *et al.* 1996, Reuter 1997, Montironi *et al.* 1998). However, in the CWR22 human prostate cancer xenograft model it was shown that the regression was due to cell cycle arrest rather than to apoptosis (Agus *et al.* 1999). It remains to be further investigated as to what degree that apoptosis is involved in tumor regression and how the process is regulated.

Progression to androgen independence after androgen-deprivation therapy is a multifactorial process by which cells acquire the ability to proliferate in the absence of androgens. Altered expression of apoptotic-regulatory genes likely plays some role in the development of hormone resistance of prostate cancer (Howell 2000). In the LNCaP prostate tumor model, adjuvant treatment with antisense Bcl-2 oligonucleotides after castration delays progression to androgen independence (Gleave *et al.* 1999). Androgen-independent prostate cancer cells also show resistance to apoptosis induction by chemotherapeutic agents and radiotherapy (Bruckheimer & Kyprianou 2000, Szostak & Kyprianou 2000). Overexpression of Bcl-2 and Bcl-XL is found in many androgen-independent cell lines and may be responsible for resistance to apoptosis (Bruckheimer & Kyprianou 2000, Coffey *et al.* 2001, Li *et al.* 2001), and antisense Bcl-2 oligonucleotides sensitize prostate cancer cells to the apoptotic effect of chemotherapeutic agents (Leung *et al.* 2001).

Retinoids and prostate cancer apoptosis

Growing evidence suggests that induction of apoptosis is a major mode of cell death in response to most cancer chemopreventive and chemotherapeutic agents (Fisher 1994, Thompson 1995, Bruckheimer & Kyprianou 2000). Retinoids exert potent apoptotic effects both in development and in cancer cells (Nagy *et al.* 1998). Retinoid-induced teratogenesis is associated with craniofacial malformations due to excessive apoptosis in the region (Sulik *et al.* 1988), while the limb malformations induced by retinoids are also associated with excessive cell death in the apical ectodermal ridge (Sulik & Dehart 1988). Retinoids regulate the development of the central nervous system in part through its apoptotic effect (Alles & Sulik 1990, 1992).

Induction of apoptosis by retinoids has been observed in various prostate cancer cells *in vitro* and *in vivo*. *Trans*-RA induces apoptosis of normal and malignant epithelial prostate cells (Pasquali *et al.* 1999), and it strongly enhances the apoptotic effect of docetaxel in DU-145 and LNCaP prostate cancer cells (Nehme *et al.* 2001). The combination of *trans*-RA and organic arsenical melarsoprol synergistically induces apoptosis of DU-145 and PC-3 cells *in vitro* and in immunodeficient mice (Koshiuka *et al.* 2000). The synthetic retinoid N-(4-hydroxyphenyl) retinamide (4HPR) is known to induce apoptosis in various malignant cells (Nagy *et al.* 1998). 4HPR also induces apoptosis of androgen-dependent and -independent cells (Sun *et al.* 1999*b*, Webber *et al.* 1999). The combination of 13-*cis* RA and phenylbutyrate synergistically induces apoptosis of several human and rodent prostate carcinoma cell lines (Pili *et al.* 2001).

The molecular mechanisms by which retinoids induce apoptosis of prostate cancer cells remain largely unknown. Induction of apoptosis of prostate cancer cells by several retinoids appears to be associated with down-regulation of Bcl-2 expression (DiPaola & Aisner 1999, DiPaola *et al.* 1999, Pasquali *et al.* 1999, Nehme *et al.* 2001), induction of insulin-like growth factor-binding protein-3 (IGFBP-3) (Goossens *et al.* 1999) and tissue transglutaminase (Pasquali *et al.* 1999), an enzyme that accumulates in cells undergoing apoptosis. Interestingly, RXRα was found to interact with IGFBP-3, and IGFBP-3-induced apoptosis was abolished in RXRα-knockout cells. It is likely that RXRα/IGFBP-3 interactions modulate the effects of IGFBP-3 on apoptosis (Liu *et al.* 2000).

RARβ and retinoid responses

The involvement of retinoid receptors in mediating proapoptotic effects of retinoids is complex, since some retinoids may act in a retinoid receptor-independent manner. However, many studies have suggested a crucial role of RARβ in the modulation of retinoid-induced apoptosis of prostate cancer cells. RARβ is up-regulated during apoptosis induced by the combination of phenylbutyrate and 13-*cis* RA in human and rodent prostate carcinoma cell lines and prostate tumors in the xenograft model (Pili *et al.* 2001), suggesting that RARβ expression may mediate the growth-inhibitory effect of retinoids. RARβ was also induced during *trans*-RA-induced apoptosis of prostate cancer cells (Pasquali *et al.* 1999). The expression of RARβ in 4HPR-treated prostate tissue was slightly higher than in the placebo-treated group (Lotan *et al.* 2000). Interestingly, introduction of RARβ in RARβ-negative prostate cancer cells resulted in increased sensitivity to the growth-inhibitory effect of retinoids and vitamin D (Campbell *et al.* 1998).

The role of RARβ in mediating the growth-inhibitory effect of retinoids was also demonstrated in many different

types of cancer cells, including breast, lung, ovarian, neuroblastoma, renal cell, pancreatic, liver, and head and neck (Nervi *et al.* 1991, Li *et al.* 1995, Hoffman *et al.* 1996, Liu *et al.* 1996, Kaiser *et al.* 1997, Xu *et al.* 1997*b*, Campbell *et al.* 1998, Ferrari *et al.* 1998, Li & Wan 1998). Expression of RARβ in RARβ-negative cancer cells restored *trans*-RA-induced growth inhibition and apoptosis, whereas inhibition of RARβ expression in RARβ-positive cancer cells abolished *trans*-RA effects (Li *et al.* 1995, Liu *et al.* 1996, Li & Wan 1998). In addition, transgenic mice expressing RARβ antisense sequences showed increased incidence of lung tumors (Berard *et al.* 1996), whereas suppression of RARβ expression was responsible for diminished anti-cancer activities of retinoids in animals (Wang *et al.* 1999). The expression of RARβ decreases as breast cells become progressively more malignant (Xu *et al.* 1997*a*), suggesting that loss of RARβ may lead to breast cancer development. Furthermore, up-regulation of RARβ is associated with a positive clinical response to retinoid in patients with premalignant oral lesions (Lotan *et al.* 1995).

The involvement of RARβ is also implicated by the finding that its expression mediates prostatic ductal branching morphogenesis in response to retinoids (Aboseif *et al.* 1997). However, expression was significantly reduced in malignant prostates compared with normal prostates (Lotan *et al.* 2000). In contrast, RARα, RARγ, RXRα and RXRγ were expressed in both normal and prostate tumor tissues (Lotan *et al.* 2000). RARβ was also selectively lost in DU-145 and PC-3 androgen-independent prostate cancer cells lines while RARα, RARγ and RXRα were well expressed (Campbell *et al.* 1998, Sun *et al.* 1999*b*). These observations suggest that loss of RARβ is associated with prostate carcinogenesis. The fact that reduced RARβ was observed in the normal tissue adjacent to the tumor suggests that this change is an early event in prostate carcinogenesis (Lotan *et al.* 2000). Similar changes were also observed in head and neck cancer (Xu *et al.* 1994).

How RARβ exerts its potent tumor-suppressive effects remains to be elucidated. A recent study demonstrated that RARβ can potently inhibit AP-1 activity (Lin *et al.* 2000*b*) and induce apoptosis of various cancer cells. The proapoptotic effect of RARβ was implicated in the finding that the expression of RARβ in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (Dolle *et al.* 1989, Mendelsohn *et al.* 1991, Ruberte *et al.* 1991, Kochhar *et al.* 1993, Soprano *et al.* 1993*a*,*b*). In our previous study, we observed that *trans*-RA-induced apoptosis in ZR-75–1 breast cancer cells is mediated by RARβ (Liu *et al.* 1996). Inhibition of RARβ activity by the expression of RARβ anti-sense RNA reduced the number of apoptotic cells, whereas *trans*-RA-induced apoptosis was only observed in hormone-independent cells when RARβ was introduced and expressed in the cells (Liu *et al.* 1996).

The mechanism that causes loss of RARβ in prostate cancer is not clear. It is unlikely that lack of RARβ expression is due to structural abnormalities of the RARβ gene (Gebert *et al.* 1991), but possibly because of changes in transcription. Expression of RARβ is highly induced by *trans*-RA through a RARE (βRARE) present in its promoter (Hoffmann *et al.* 1990, Sucov *et al.* 1990, de The *et al.* 1990), which is activated by RAR/RXR heterodimers in response to retinoids (Zhang *et al.* 1992*a*). Vitamin A serum levels are lower in patients with prostate cancer (Reichman *et al.* 1990). In addition, prostate cancer tissues have five to eight times less *trans*-RA than normal prostate or benign prostate (Pasquali *et al.* 1996). Reduced levels of retinoids in prostate cancer tissue may contribute to loss of RARβ expression. Interestingly, RARβ cannot be induced by exogenous retinoids in androgen-independent prostate cancer cells, despite expression of RARs and RXRs in these cells (Sun *et al.* 1999*b*). Similar observations were also made in other cancer, such as lung cancer, cells which express RARs and RXRs, but fail to express RARβ in response to retinoids (Zhang *et al.* 1994). These observations argue against the involvement of reduced retinoid levels in inhibiting RARβ expression, and also demonstrate that expression of RARs and RXRs is not sufficient to render RARβ expression responsive to *trans*-RA. Thus, factors other than RARs and RXRs are required for the effect of *trans*-RA on inducing RARβ expression, and these may be lost in cancer cells. Recently, we found that expression of the orphan receptor COUP-TF is positively correlated with RARβ induction and growth inhibition by *trans*-RA in various cancer cell lines and it is underexpressed in many RARβ-negative cancer cell lines (Wu *et al.* 1997*b*, Lin *et al.* 2000*a*). Further studies demonstrated that COUP-TF is required for *trans*-RA to induce RARβ expression, growth inhibition and apoptosis in cancer cells (Lin *et al.* 2000*a*). The effect of COUP-TF is likely due to its transactivation of the RARβ promoter through its binding to a DR-8 element present in the promoter, resulting in enhanced interaction of RARα with its co-activator CBP (Lin *et al.* 2000*a*). Thus, COUP-TF induces RARβ promoter transcription by acting as an accessory protein for RARα to recruit its co-activator. Whether lack of COUP-TF expression is responsible for loss of RARβ in androgen-independent prostate cancer cells remains to be illustrated. Methylation of the RARβ promoter was recently reported to contribute to RARβ inactivity (Sirchia *et al.* 2000), suggesting a possibility of hypermethylation of the RARβ promoter in prostate cancer cells.

The anti-cancer effects of conventional retinoids appear to be limited to androgen-dependent prostate cancer cells, whereas the more aggressive, androgen-independent prostate cancer cells are refractory (Campbell *et al.* 1998). Loss of RARβ induction by *trans*-RA may be responsible for diminishment of *trans*-RA activities in androgen-independent prostate cancer cells. Induction of RARβ by classical retinoids,

such as *trans*-RA, is mediated by activation of RAR/RXR heterodimers which bind to the βRARE (Zhang *et al.* 1992*a*). Unfortunately, this pathway appears to be impaired in androgen-independent prostate cancer cells. It is therefore important to identify alternative pathways that activate the RARβ promoter. Recent studies have demonstrated that RXR-selective retinoids represent promising agents for the prevention and treatment of cancer. 9-*cis* RA has demonstrated significant anti-proliferative and/or differentiating activity in *in vitro* models of breast cancer (Anzano *et al.* 1994, Rubin *et al.* 1994, Gottardis *et al.* 1996*b*), leukemia and lymphoma (Gottardis *et al.* 1996*b*), lung cancer (Guzey *et al.* 1998), and head and neck cancer (Giannini *et al.* 1997). Its activity was also observed in prostate cancer cells (Blutt *et al.* 1997, McCormick *et al.* 1999). Combination of 9-*cis* RA and 1,25-dihydroxyvitamin D3 synergistically inhibited the growth of LNCaP (Blutt *et al.* 1997, McCormick *et al.* 1999). McCormick *et al.* (1999) conducted a chemoprevention study to evaluate the activity of 9-*cis* RA as an inhibitor of prostate carcinogenesis in animals, and observed that continuous dietary administration of 9-*cis* RA before MNU administration reduced cancer incidence in the dorsolateral+anterior prostate. Similarly, the dosage levels of 9-*cis* RA reduced the incidence of cancer in all accessory sex glands (McCormick *et al.* 1999). RXR-selective retinoids were more effective than *trans*-RA at inhibiting mammary carcinogenesis in animals (Anzano *et al.* 1994), and RXR-selective retinoid LGD 1069 inhibited the growth of established breast tumors (Gottardis *et al.* 1996*a*, Bischoff *et al.* 1998).

How RXR ligands effectively inhibit the growth of cancer cells has not been established. Through its binding to RXR, RXR ligands may indirectly influence a wide range of functions, which are regulated by other nuclear receptors that heterodimerize with RXR (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). In our previous studies (Wu *et al.* 1997*a*), we observed that inhibition of cancer cell growth by RXR-selective retinoids was associated with induction of RARβ expression in estrogen-independent MDA-MB231 cells and lung cancer cells (Wu *et al.* 1997*a*), suggesting that induction of RARβ expression contributes to the growth-inhibitory effects of these retinoids. Furthermore, we observed that their effect on RARβ induction is in part mediated through TR3/RXR heterodimers which bind to the βRARE (Wu *et al.* 1997*a*). Thus, RXR ligands may exert their potent anti-cancer activity through inducing RARβ expression in cancer cells that are resistant to classical retinoids (Fig. 3). Thus, specific ligands for the RXR receptor may have significant activity as inhibitors of carcinogenesis in the prostate, whereas retinoids whose binding is limited to RAR may be inactive.

AHPN and its analogs: potent apoptotic inducers of prostate cancer cells

The sensitivity of prostate cancer cells to apoptosis-inducing effects of retinoids diminishes during the progression of prostate tumors. Androgen-independent derivatives of LNCaP cells were more resistant than their parental androgen-dependent LNCaP cells to apoptotic effects of *trans*-RA. In addition, malignant prostate cancer cells showed resistance to radiotherapy and chemotherapy. This has been the major challenge in the therapy of prostate cancer. Thus, retinoids capable of inducing apoptosis of advanced malignant prostate cancer cells are expected to be suitable agents for prostate cancer treatment.

Recently, a new class of synthetic retinoids related to AHPN (also called CD437) (Bernard *et al.* 1992) has been found to potently inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate carcinoma cells (Liang *et al.* 1999, Lu *et al.* 1999, Li *et al.* 2000, Sun *et al.* 2000). When the growth-inhibitory and apoptosis-inducing effects of *trans*-RA and AHPN were compared in androgen-dependent and -independent prostate cancer cell lines, AHPN significantly inhibited the growth and induced apoptosis of androgen-independent prostate cancer cell lines, while *trans*-RA had little effect on these cells (Sun *et al.* 2000). A synthetic retinoid, CD-271, which is related to AHPN and selectively activates the RARγ subtype in a given context, also shows increased anti-proliferative activity against prostate cancer cells over *trans*-RA (Lu *et al.* 1999). Interestingly, AHPN was more effective in killing androgen-independent cells such as DU-145 and PC-3 than the androgen-dependent LNCaP cells (Sun *et al.* 2000). Thus, AHPN may be representative of a novel class of compounds suitable for treatment of androgen-independent prostate cancer. AHPN was also identified to be a potent apoptosis inducer in many different types of cancers, including lung (Sun *et al.* 1997, 1999*c*,*d*,*e*, Adachi *et al.* 1998*b*, Li *et al.* 1998), cervical (Oridate *et al.* 1997), ovarian (Langdon *et al.* 1998), melanoma (Schadendorf *et al.* 1995, 1996), leukemia (Hsu *et al.* 1997, Gianni & de The 1999, Mologni *et al.* 1999) and neuroblastoma (Meister *et al.* 1998). The apoptotic effect of AHPN is independent of retinoid receptor expression, indicating that its activity is not restricted by lack of RARβ in prostate cancer cells.

Orphan receptor TR3: a regulator of both survival and apoptosis of prostate cancer cells

AHPN-induced apoptosis may involve p53-dependent and -independent as well as caspase-dependent and -independent pathways (Adachi *et al.* 1998*a*, Fontana *et al.* 1998, Hsu *et al.* 1999, Marchetti *et al.* 1999, Zhang *et al.* 1999, Zhang 2000). Expression of a variety of apoptosis-associated genes, such as cJun, cFos, c-Myc, p21, Bcl-2, Bax, DR4, DR5 and Fas can be regulated by AHPN in a cell type-specific manner. Their role in AHPN-induced apoptosis remains to be determined. We have recently demonstrated that the expression of

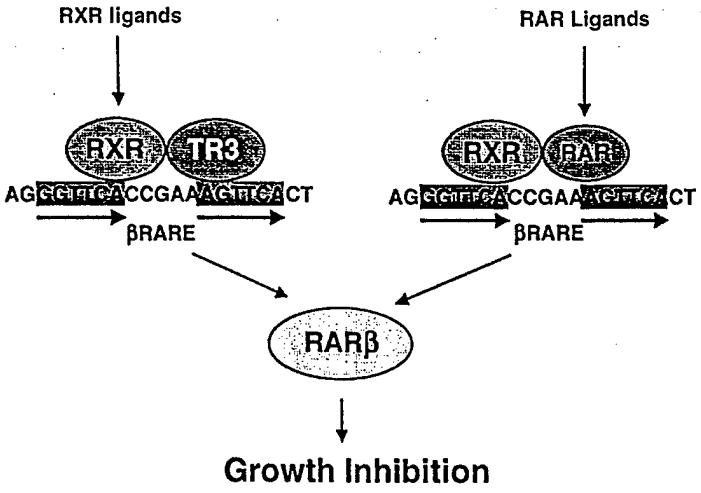

Figure 3 Signaling pathways for RARβ induction. βRARE in the RARβ promoter is essential for induction of RARβ by retinoids. The βRARE can be activated by RAR/RXR heterodimer in response to RAR ligands. Alternatively, it can be activated by RXR ligands through a TR3/RXR heterodimer that also binds to the βRARE.

TR3 is required for AHPN-induced apoptosis in human prostate cancer cells (Li *et al.* 2000). TR3 message was ~~also~~ highly induced by AHPN in LNCaP cells (Li *et al.* 2000). The apoptotic effect of the AHPN analog MM11453 was completely abolished in LNCaP cells stably expressing TR3 antisense RNA (Li *et al.* 2000).

TR3 (Chang & Kokontis 1988, Hazel *et al.* 1988, Milbrandt 1988) is an immediate early response gene whose expression is rapidly induced by a variety of growth stimuli, including growth factors, phorbol ester and cAMP-dependent pathways (Chang & Kokontis 1988, Hazel *et al.* 1988, Milbrandt 1988, Fahrner *et al.* 1990, Wilson *et al.* 1993, Crawford *et al.* 1995, Lim *et al.* 1995). It is also an orphan member of the steroid/thyroid/retinoid receptor superfamily (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). Like other immediate early growth response genes, such as c-myc and c-jun, TR3 plays a role in controlling cell proliferation and mediating apoptosis (Bravo 1990, Herschman 1991). TR3 is rapidly induced during apoptosis in immature thymocytes and T-cell hybridomas (Liu *et al.* 1994, Woronicz *et al.* 1994). Overexpression of a dominant negative TR3 protein (Woronicz *et al.* 1994) or inhibition of TR3 expression by antisense TR3 inhibits apoptosis in thymocytes (Liu *et al.* 1994), whereas constitutive expression of TR3 results in massive apoptosis (Xue *et al.* 1997).

TR3 plays a critical role in regulating both proliferation and apoptosis of prostate cancer cells. Levels of TR3 are dramatically induced by androgen (Uemura & Chang 1998) and epidermal growth factor (Li *et al.* 2000) in LNCaP prostate cancer cells ~~as well as by androgen ablation~~ and in the ventral prostate of animals (Uemura & Chang 1998). Interestingly, TR3 is also rapidly induced in LNCaP cells in response to apoptotic stimuli, including AHPN (Li *et al.* 2000), calcium ionophore, etoposide (VP-16) (Uemura & Chang 1998, Li *et al.* 2000) and phorbol ester (Young *et al.* 1994, Li *et al.* 2000). Expression of TR3 antisense RNA significantly inhibits apoptosis induced by these agents (Li *et al.* 1998, Uemura & Chang 1998). Because of its potent effects in regulating cellular proliferation and apoptosis, TR3 may play a role in the development or progression of prostate cancer. In fact, TR3 is more highly expressed in prostate cancer areas than in adjacent normal or benign prostate hypertrophic tissue (Uemura & Chang 1998). TR3 is also highly expressed in lung cancer cell lines (Wu *et al.* 1997*b*). The role of TR3 in cancer development is further indicated by the finding that TR3 is involved in a chromosomal translocation identified in extra-skeletal myxoid chondrosarcoma (Labelle *et al.* 1995, 1999).

How TR3 exerts opposing biological activities is poorly understood. Similar to other members of the steroid/thyroid/retinoid receptor superfamily, it was believed that TR3 functioned in the nucleus as a transcriptional factor to regulate gene expression necessary to alter the cellular phenotype in response to various stimuli. TR3 response elements (NBRE or NurRE) have been identified (Wilson *et al.* 1991, Philips *et al.* 1997). In addition, TR3 can heterodimerize with RXR (Forman *et al.* 1995, Perlmann & Jansson 1995, Wu *et al.* 1997*a*) and COUP-TF (Wu *et al.* 1997*b*). The observations that over-expression of TR3 in cancer cells confers retinoid resistance by modulating transcriptional regulation of retinoids (Wu *et al.* 1997*b*) and that the TR3 fusion protein identified in extra-skeletal myxoid chondrosarcoma is about 270-fold more active than the native receptor in transactivation (Labelle *et al.* 1995, 1999) suggests that TR3 may mediate cell proliferation through its transcriptional regulation.

Much less is known about the mechanism by which TR3 functions to regulate apoptosis. TR3 might be involved in the apoptotic process by regulating expression of certain apoptosis-associated genes (Liu *et al.* 1994, Woronicz *et al.* 1994, 1995, Weih *et al.* 1996, Cheng *et al.* 1997). Unfortunately, no comprehensive characterization of its target genes has been achieved. By using a variety of approaches, we recently demonstrated that TR3-dependent apoptosis of LNCaP prostate cancer cells does not require its DNA binding and transactivation, but is associated with translocation of this protein from the nucleus to mitochondria, where it resides on the outer mitochondrial membrane and induces cytochrome c release (Li *et al.* 2000). These results reveal a novel mechanism by which a nuclear transcriptional factor translocates to mitochondria to initiate apoptosis (Fig. 4). Translocation of TR3 between the nucleus and the cytoplasm represents a new mechanism for cross-talk between different signaling pathways (Fig. 4). This exciting finding, together with the observations that TR3 is associated with cancer cell proliferation by acting as a nuclear transcriptional factor, demonstrates that the opposing biological activities of TR3 are regulated by its subcellular localization. These data suggest a new approach of eliminating prostate cancer cells by inducing cytoplasmic localization of TR3. AHPN analogs and other agents that specifically induce TR3 mitochondrial localization will not only induce apoptosis of prostate cancer cells mediated by TR3 mitochondrial action but also inhibit cancer cell proliferation induced by androgen or growth factors through nuclear action of TR3.

Prospective

Induction of apoptosis is an effective way to eliminate cancer cells. The acquisition of resistance toward apoptosis during prostate tumor progression is perhaps the major obstacle in the treatment of prostate cancer. Retinoids inhibit the growth and induce apoptosis of prostate cancer cells *in vitro* and prevent prostate carcinogenesis in animals, suggesting that retinoids are promising agents for the prevention and treatment of human prostate cancer. However, the apoptotic effect of classical retinoids diminishes in androgen-independent prostate cancer cells, and clinical trials using conventional retinoids have not demonstrated significantly beneficial effects. Loss of RARβ may contribute to retinoid resistance in advanced prostate cancer cells. Alternative approaches to induce RARβ expression may render prostate cancer cells sensitive to apoptotic effects of retinoids. *In vitro* and animal studies have suggested that RXR ligands are effective inhibitors of prostate carcinogenesis and they are capable of inducing RARβ expression through alternative approaches, such as TR3/RXR heterodimers. Elucidation of their mechanisms of action will provide valuable information, allowing design and identification of a new generation of synthetic retinoids that are likely to be more effective in the prevention and treatment of prostate cancer.

Synthetic retinoids related to AHPN effectively induce apoptosis of both androgen-dependent and -independent prostate cancer cells, indicating that these retinoids represent a new class of drugs that have therapeutic value for the treatment of prostate cancer. The clinical potential of this class of retinoids and their new generation needs to be explored.

Modern biology has suggested that cancer drug discovery based on molecular differences between tumor and normal cells is a new and feasible approach. With an improved understanding of apoptotic processes in prostate cancer cells, many potential new targets for therapy can be discovered. The illustration that orphan receptor TR3 mediates the apoptotic effect of AHPN analogs in prostate cancer cells suggests that TR3 is an ideal target for cancer drug development. Levels of TR3 are induced by androgen and growth factor in prostate cancer cells as well as by androgen ablation and may be necessary to support proliferation of prostate cancer cells. Thus, TR3 can mediate opposing biological activities, cell death and survival (Fig. 4). The unique property of TR3 provides an excellent opportunity to develop novel drugs targeted at TR3. Agents such as AHPN and its analogs that specifically induce mitochondrial localization of TR3 will convert TR3 from a cancer cell-promoting (adverse effect) to a cancer cell apoptosis-inducing (beneficial effect) molecule.

Cellular localization of TR3 defines its biological function. How TR3 is translocated from the nucleus to the cytoplasm and targets mitochondria in response to apoptotic stimuli is unclear. This information is essential for developing retinoids that induce mitochondrial localization of TR3. The fact that TR3 mitochondrial targeting is regulated by various stimuli, including TPA, calcium ionophore and growth factors (Li *et al.* 2000), which are known to act through membrane signaling pathways involving various kinases and phosphatases, suggests that phosphorylation of TR3 may play a crucial role in regulating TR3 subcellular activities.

The observation that TR3 can heterodimerize with RXR (Forman *et al.* 1995, Perlmann & Jansson 1995, Wu *et al.* 1997*a*) suggests that RXR and its ligands are likely involved in the regulation of TR3-dependent apoptotic pathways. This is supported by previous observations that RXR and its ligand 9-*cis*-RA inhibit activation-induced apoptosis of T-cells and thymocytes (Yang *et al.* 1993, 1995*a*,*b*, Bissonnette *et al.* 1995, Szondy *et al.* 1998), in which TR3 plays a role (Liu *et al.* 1994, Woronicz *et al.* 1994, 1995). RXR, through its heterodimerization with TR3, may be required for cytoplasmic localization of TR3 or for its mitochondrial targeting. Illustrating the molecular mechanisms by which RXR and its ligands regulate TR3-dependent apoptotic pathways in prostate cancer cells will provide additional modes to regulate apoptosis of prostate cancer cells and new treatment approaches for prostate cancer.

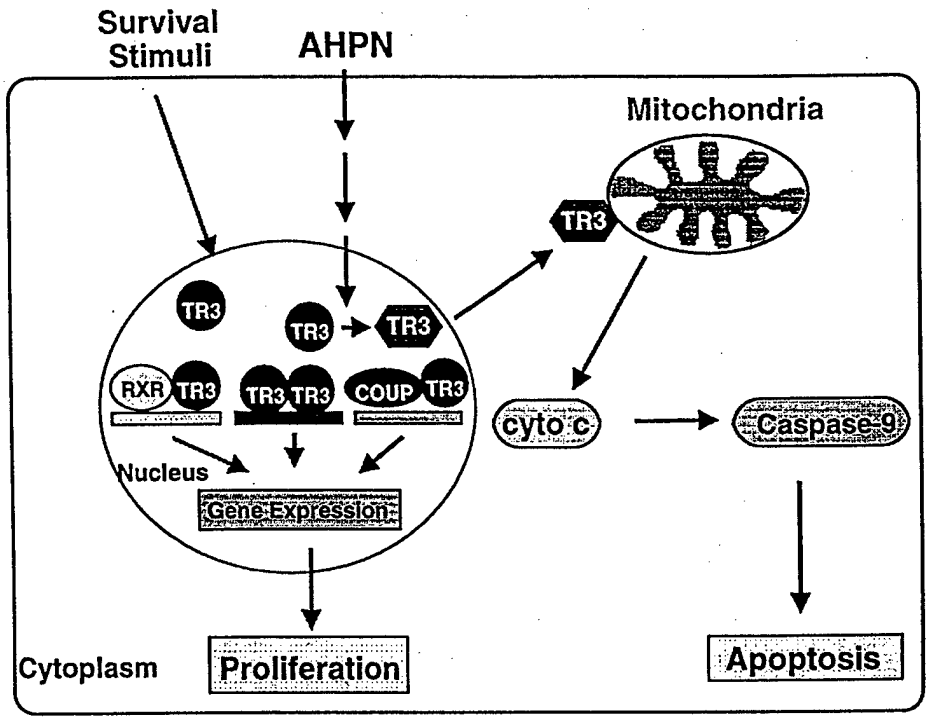

Figure 4 TR3-dependent cell survival and cell death pathways. TR3 induced by survival stimuli, such as growth factors, functions in the nucleus through either its homodimerization or heterodimerization with RXR or COUP-TF to regulate expression of genes involved in cell proliferation. In contrast, TR3 induced by death stimuli, including AHPN, may undergo a conformational change, which is required for its export to the cytoplasm, where it resides on mitochondria. On mitochondria, TR3 regulates mitochondrial activities, resulting in release of cytochrome c (cyto c) into the cytosol.

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